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2,325,765, on November 2, 2000, by LISA McKERRACHER, for "Method for Making  
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O P I C  C I P O

**mETHODS FOR MAKING AND DELIVERING Rho-antagonist TISSUE ADHESIVE FORMULATIONS to the injured mammalian central AND PERipheral nervous systems AND USES THEREOF**

**ABSTRACT**

5      The present invention provides methods for making, delivering and using formulations that combine a therapeutically active agent(s) (such as for example a Rho antagonist(s)) and a flowable carrier component capable of forming a therapeutically acceptable matrix *in vivo* (such as for example tissue adhesives), to injured nerves to promote repair and regeneration and regrowth of injured mammalian neuronal cells, e.g. for facilitating axon growth at a desired  
10     lesion site. Preferred Rho antagonists are C3, chimeric C3 proteins, or trans-4-amino(alkyl)-1-pyridylcarbamoylcyclohexane compounds as Rho kinase inhibitors. The system for example may deliver an antagonist(s) in a tissue adhesive such as a fibrin glue or a collagen gel to create a delivery matrix *in situ*. A kit and methods of stimulating neuronal regeneration are also included.

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METHODS FOR MAKING AND DELIVERING Rho-antagonist TISSUE ADHESIVE FORMULATIONS to the injured mammalian central AND PERipheral nervous systems AND USES THEREOF

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**FIELD OF THE INVENTION**

The present invention pertains to the field of mammalian central nervous system repair, axon regeneration and axon sprouting. The present invention relates to a method of delivery of C3 or other Rho antagonists to repair damage in the nervous system. The invention also pertains to use of the delivery system for toxicity testing of compounds applied to the injured CNS.  
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**BACKGROUND**

**Repair, regeneration and regrowth in the CNS.**

Traumatic injury of the spinal cord results in permanent functional impairment. Most of the deficits associated with spinal cord injury result from the loss of axons that are damaged in the central nervous system (CNS). Similarly, other diseases of the CNS are associated with axonal loss and retraction, such as stroke, HIV dementia, prion diseases, Parkinson's disease, Alzheimer's disease, multiple sclerosis and glaucoma. Common to all of these diseases is the loss of axonal connections with their targets, and the ability to stimulate growth of axons from the affected or diseased neuronal population would improve recovery of lost neurological functions. For example, following a white matter stroke, axons are damaged and lost, even though the neuronal cell bodies  
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are alive. Treatments that are effective in eliciting sprouting from injured axons are equally effective in treating some types of stroke (Boston life sciences, Sept. 6, 2000 Press lease [www.bostonlifesciences.com](http://www.bostonlifesciences.com)). Similarly, although the subject of this invention is related to delivery of Rho antagonists to the traumatically damaged nervous system, this invention also pertains to damage from unknown causes, such as during multiple sclerosis, HIV dementia, Parkinson's disease, Alzheimer's disease, prion diseases or other diseases of the CNS were axons are damaged in the CNS environment.

At present there are no clinical treatments available to stimulate regeneration of cut axons. Numerous studies now show that anatomical regeneration and functional recovery are possible in rodent models of spinal cord injury. Clinical treatments that foster regeneration would be a significant improvement over present treatments that serve only to limit the extent of secondary damage caused by non-neuronal cells that invade the injury site. Spinal cord injured patients receive systemically high doses of the steroid methylprednisolone immediately following injury to suppress an unfavorable inflammatory reaction. This drug, however, does not restore functions that are lost when axons are cut.

Several major advances in our understanding of axon regeneration have led to the ability to stimulate some axon regeneration and functional repair in animal models of spinal cord injury. In the 1980's experiments by Aguayo and colleagues to use peripheral nerve grafts that were inserted into the brain or spinal cord showed that CNS neurons have the capacity to regrow, and these studies highlighted that diverse classes of CNS neurons have the potential to regenerate when given a permissive growth environment (Aguayo, et al. (1981)J Exp Biol.95:231-40). However, this technique cannot be used to rewire the complex circuitry of the CNS. Another major advance in our understanding of axon regeneration in the central nervous system was the discovery by Schwab and colleagues that the CNS environment did not simply lack growth promoting molecules, but that growth inhibitory molecules existed to block axon growth (Schwab, et al. (1993)Annu.Rev.Neurosci.16:565-595). Long distance regeneration in the CNS by blocking growth inhibitory molecules with antibodies was first achieved in juvenile rats by neutralization of inhibitory protein activity with the IN-1 antibody in spinal cord (Schnell and Schwab (1990)Nature.343:269-272) and optic nerve (Weibel, et al. (1994)Brain Res.642:259-266). However, this technique suffers from the problem that only a single growth inhibitory protein is targeted, and delivery by the application of hybridoma cells or by infusing antibodies with pumps. There have been investigations on the use of growth factors to promote regeneration in the CNS, some with notable success (Ramer, et al. (2000)Nature.403:312-316, Liu, et al. (1999)J Neurosci.19:4370-87, Blesch, et al. (1999)J Neurosci.19:3556-66). Typically infusion pumps or gene therapy techniques are used to deliver growth factors to injured neurons. In general, trophic factors do not stimulate long distance regeneration, but stimulate more of a local sprouting response (Schnell, et al. (1994)Nature.367:170-173, Mansour-Robaey, et al. (1994)Proc.Natl.Acad.Sci.91:1632-1636).

A more recent advance is the demonstration that increasing the intrinsic growth capacity of neurons is sufficient to allow axon regeneration in the CNS, and that neurons primed for regeneration with neurotrophins, a conditioning lesion, or treatment with Rho antagonists have a better chance to grow on inhibitory substrates (Neumann (1999)Neuron.23:83-91, Cai, et al. (1999)Neuron.22:89-101, Lehmann, et al. (1999)J. Neurosci.19:7537-7547). Targeting intracellular signalling mechanisms is likely to be the most efficient way to promote axon regeneration, and we have found that Rho antagonists are able to stimulate regeneration in the optic nerve of adult rats

(Lehmann et al (1999) IBID). However, our preliminary experiments to apply Rho antagonists to the injured spinal cord were not successful. Likely the infused protein was not sufficiently retained at the injury site, either by syringe application or the use of Gelfoam. This suggested to us that the delivery of compounds that act with low affinity (compared to high affinity neurotrophins) posed unique problems in delivery. We have developed a tissue-adhesive delivery system whereby the Rho antagonist is added to the adhesive solution before application of the solution with a syringe, and polymerization of the adhesive within the lesion cavity in the CNS.

**Repair, regeneration and regrowth in the peripheral nervous system (PNS).**

While neurons in the peripheral nervous system regenerate naturally, there are many techniques used to enhance and help the repair process. Most of these techniques are not aimed at stimulating the rate of axonal regeneration, but in helping to guide axons back towards their target regions. For example, severed nerve are sewn or glued together with a fibrin glue enhance the repair process. While our invention is directed at repair in the CNS, an extension of its use would be in PNS repair. Treatment with Rho antagonists in the adhesive delivery system could be used to enhance the rate of axon growth in the PNS. This is first use of Rho antagonists in the PNS.

**Targeted delivery of drug that promote regeneration in the CNS and PNS**

Different methods have been used for local delivery of drugs in the CNS, however none of these methods have been developed as a kit with biological component that have proven effective in the promotion of the regeneration of injured axons. IN-1 is an antibody that promotes regeneration in the CNS. One method of delivery is the implantation of cells that secrete the active antibody (Schnell et al (1994) Nature 367:170). The use of fibrin adhesive for the delivery of IN-1 antibody was not found to be effective. (Guest (1997)J. Neurosci. Res.50:888-905). Another method is the use of pumps to infuse and deliver continuously over time compounds that stimulate regeneration. (Ramer, et al. 2000. Nature. 403:312-316, Verge, et al. 1995. Journal of Neuroscience. 15:2081-2096);

Fibrin adhesives have been used in studies of CNS regeneration. It has been used in replacement of sutures to graft peripheral nerves into the damaged CNS (Cheng, et al. (1996)Science.273:510-513). A fibrin glue has also been used for the delivery of fibroblast growth factor (FGF) to damaged corticospinal neurons (Guest (1997)J. Neurosci. Res.50:888-905). The use of fibrin glue plus FGF did not promote long distance regeneration.

Collagen has been tested for its ability to promote regeneration after injury. (Joosten (1995)J. Neurosci. Res.41:481-490.). Collagen has also been used for the delivery of neurotrophins to injured corticospinal axons (Houweling (1998)Expt. Neurol.153:49-59). Neither of the

conditions was able to support long distance regeneration. In tissue culture, collagen gels can maintain gradients of small molecules important in axon guidance (Kennedy, et al. (1994) *Cell*.**78**:425-435). Moreover, it had been reported that collagen gels by themselves could foster some axon regeneration after spinal cord injury (Joosten (1995) *J. Neurosci. Res.* **41**:481-490.). We found that the combination of collagen gels and C3 was able to allow axons to into the site of the glial scar. Based on our experiments with fibrin glue (see below), we predict that delivery of C3 in collagen would be improved by the addition of protease inhibitors to prevent lysis of the gel and C3.

#### 10 Types of Rho antagonists and uses.

Growth inhibitory proteins cause growth cone collapse (Li, et al. (1996) *J. Neurosci. Res.* **46**:404-414, Fan, et al. (1993) *J. Cell Biol.* **121**:867-878) and it has become clear that GTPases of the Rho family that comprise Rho, Rac and Cdc42 are intracellular regulators of growth cone collapse (Lehmann, et al. (1999) *J. Neurosci.* **19**:7537-7547,

15 Tigyi, et al. (1996) *Journal of Neurochemistry*.**66**:537-548, Kuhn, et al. (1999) *J. Neurosci.* **19**:1965-1975, Jin and Strittmatter (1997) *J. Neurosci.* **17**:6256-6263). These small GTPases exist in inactive (GDP) and active (GTP) forms, and the cycling between active GTP-bound and inactive GDP-bound states is tightly regulated. The guanine nucleotide exchange factors (GEFs) accelerate the release of GDP, thereby facilitating GTP binding. The GTPase activating proteins (GAPs) catalyze GTP hydrolysis and conversion of the inactive form. The GDP dissociation inhibitors (GDIs) act to maintain Rho in a GDP-bound form. GEFs for Rho all have a domain homologous with the Dbl oncprotein, and more than 20 such proteins have been identified, including Tiam-1 which is highly expressed in brain (Zheng and Li (1999) *J. Biol. Chem.* **272**:4671-4679, van Leeuwen, et al. (1997) *J. Cell Biol.* **139**:797-807). Once in the active form, Rho GTPases typically stimulate ser/thr kinases, such as ROK (Rho kinase), PAK (p21-activated kinase) and downstream effectors that act on the cytoskeleton.

20 The Rho family members that regulate the cytoskeleton and motility include Rho, Rac and Cdc42 (Nobes and Hall (1995) *Cell* **1995**.*81*:53-62). Rho is an important link between signaling through integrins and signaling cascades of trophic factors (Laudanna, et al. (1996) *Science*.**271**:981-983, Hannigan, et al. (1996) *Nature*.**379**:91-96, Kuhn, et al. (1998) *J. Neurobiol.* **37**:524-540). Cdc42 is important for the regulation of filopodia (Nobes and Hall (1995) *Cell* **1995**.*81*:53-62). Both Rac and Rho regulate growth cone motility and axon growth. In non-neuronal cells a hierarchy of signaling between Rho, Rac and Cdc42 exists (Hall (1996) *Ann. Rev. Cell Biol.* **10**:31-54). In neurons Rac and Rho may have opposite effects (van Leeuwen, et al. (1997) *J. Cell Biol.* **139**:797-807, Kozma, et al. (1997) *Molec. Cell. Biol.* **17**:1201-1211). Activation of Rac stimulates outgrowth of neurites from N1E-115 neuroblastoma neurons whereas activation of Rho causes neurite retraction (van Leeuwen, et al. (1997) *J. Cell Biol.* **139**:797-807, Albertinazzi, et al. (1998) *J. Cell Biol.* **142**:815-825). In PC12 cells, dominant negative Rac disrupts neurite outgrowth in response to NGF (Hutchens, et al. (1997) *Molec. Biol. Cell*.**8**:481-500, Daniels, et al. (1998) *EMBO Journal*.**17**:754-764) whereas

treatment of PC12 cells with lysophosphatidic acid (LPA), a mitogenic phospholipid that activates Rho, causes neurite retraction (Tigyi, et al. (1996) *Journal of Neurochemistry*.**66**:537-548). The p21-activated kinase (PAK) is activated by Rac, and PAK can also induce PC12 cell neurite outgrowth (Daniels, et al. (1998) *EMBO Journal*.**17**:754-764). We have shown that inactivation of Rho is sufficient to promote PC12 cell neurite outgrowth on growth inhibitory substrates (Lehmann, et al. (1999) *J. Neurosci.***19**:7537-7547). A recent study of activating and null mutations of Rho expressed in PC12 cells suggests that the differentiation state is an important parameter for the effect of Rho on neurite outgrowth, and that priming PC12 cells with NGF can alter the responsiveness to activating and null mutations. (Sebok, et al. (1999) *J. Neurochem.***73**:949-960). This result is in agreement with the finding that priming neurons increases intracellular cAMP (Cai, et al. (1999) *Neuron*.**22**:89-101), which can in turn influence the activation of Rho. (Lang, et al. (1996) *EMBO J.***15**:510-519, Dong, et al. (1998) *J. Biol. Chem.***273**:22554-22562).

In primary neurons Rac and Rho regulate both dendrite and axon growth and cone morphology and collapse. By immunocytochemistry we demonstrated that Rho is concentrated in growth cones, and some colocalizes at sites of point contact. (Renaudin, et al. (1998) *J. Neurosci. Res.***55**:458-471). Experiments with activating and dominant negative mutations have demonstrated that activation of Rac is important in maintaining a spread morphology after challenge with growth cone collapsing factors. (Kuhn, et al. (1999) *J. Neurosci.***19**:1965-

1975, Jin and Strittmatter (1997) *J. Neurosci.***17**:6256-6263). The activation of Rho induces growth cone collapse, and collapse can be prevented by treatment with *Clostridium botulinum* C3 exo-Transferase (hereinafter simply referred to as C3) (Tigyi, et al. (1996) *Journal of Neurochemistry*.**66**:537-548, Jin and Strittmatter (1997) *J. Neurosci.***17**:6256-6263). C3 inactivates Rho by ADP-ribosylation and is fairly non-toxic to cells. (Dillon and Feig

(1995) *Methods in Enzymology: Small GTPases and their regulators Part. B*.**256**:174-184).

An important downstream target of activated Rho is p160ROK, a Rho kinase. (Kimura and Schubert (1992) *Journal of Cell Biology*.**116**:777-783, Keino-Masu, et al. (1996) *Cell*.**87**:175-185, Matsui, et al. (1996) *EMBO J.***15**:2208-2216, Matsui, et al. (1998) *J. Cell Biol.***140**:647-657, Ishizaki (1997) *FEBS Lett.***404**:118-124). Among other effects, ROK phosphorylates myosin phosphatase to regulate actin-myosin based motility. (Matsui, et al. (1996) *EMBO J.***15**:2208-2216) and regulates proteins of the ezrin family (Vaheri, et al. (1997) *Curr. Opin. Cell Biol.***9**:659-666), which are concentrated in neuronal growth cones. (Goslin, et al. (1989) *J. Cell Biol.***109**:1621-1631). Activation of ROK also induces growth cone collapse,

which can be prevented by the addition of the ROK inhibitor Y-27632 ENRfu(Hirose, et al. (1998)J. Cell Biol. 141:1625-1636).

The above studies showed that Rho antagonists can stimulate regeneration in the CNS. It has been demonstrated that Rho kinase is an important downstream target of Rho signaling (Matsui, et al. (1996)EMBO J. 15:2208-2216, Bito (2000)Neuron. 26:431-441). Among other effects, inactivation of Rho kinase stimulates neurite outgrowth in tissue culture (Bito (2000)Neuron. 26:431-441) as does inactivation of RhoENRfu(Lehmann, et al. (1999)J. Neurosci. 19:7537-7547). Therefore, inactivation of Rho kinase should induce the same biological effects *in vivo* as inactivation of Rho.

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### **Y-27632**

The Rho kinase inhibitory Y-27632 is a trans-4-amino(alkyl)-1-pyridylcarbamoylcyclohexane compound (US patent no. 4,997,834); please see also Ishizali et al. 2000. Molecular Pharmacology 57:976-983 which refers to Y-27632 in the dihydchloride form as well as to a related compound, namely (R)-trans--4-(1-aminoethyl)- N-(1H-pyrrolo[2,3]pyridin-4-yl) cyclohexanecarboamide dihydchloride. A patent application "Medicines comprising Rho kinase inhibitor" has been submitted (EPO 956 865 A1). This inhibitor has not been tested for efficacy in CNS injury, nor has the company who patented this compound discovered how it might be applied to a region of CNS injury in a kit form. Such a kit is provided in our invention. Please see also European Patent application European application #97934756.4 PCT/JP97/02793 International publication # WO 98/06433 (19.02.1998/07).

25 The compound (R)-(+)-trans-N-(4-pyridyl)-4-(1-aminoethyl)- cyclohexanecarboamide (Y-27632) inhibits Rho kinase at sub-micromolar concentrations ENRfu(Uehata, et al. (1997)Nature. 389:990-994). Y-27632, made by a Yoshitoma, affects calcium sensitization of smooth muscles to affect hypertension. It was reported that the cellular target of Y-27632 is Rho-associated protein kinase, p160ROCK. (Uehata, et al. (1997)Nature. 389:990-994, Somlyo (1997)Nature. 389:908-911).

30 With the *in vitro* evidence that Rho was a key protein to regulate the response to growth inhibitory molecules, we tested the ability of C3 to stimulate regeneration *in vivo*. We crushed adult rat optic nerves and applied C3 at the same time, directly at the lesion site. (Lehmann, et al. (1999)J. Neurosci. 19:7537-7547). We found that large numbers of axons traversed the lesion to grow in the distal optic nerve. For these experiment the C3 was delivered to optic nerve through the

use of gelfoam an Elvax, a slow release matrix . . . (Lehmann, et al. (1999) *J. Neurosci.* 19:7537-7547). Our current invention is a delivery system and kit that has been adapted to apply C3, chimeric C3, or Rho kinase inhibitor to injured regions of the CNS that include injured spinal cord or brain, and regions of the CNS injured by stroke.

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#### Tissue Adhesives types

Many different protein-based tissue adhesives exist Examples include collagen gels, fibrin tissue adhesives, matrigel™, laminin networks, and adhesives based on a composition of basement membrane proteins that contain collagen. Perhaps the most popular are the fibrin 10 adhesives.

Fibrin sealant has three basic components: fibrinogen concentrate, calcium chloride and thrombin. Other components can be added to affect the properties of the gel formation. Added components are used to modulate time it takes for the fibrin gel to form from the soluble components, the size of the protein network that is formed, the strength of the gel, and protease 15 inhibitors slow down the removal of the gel after it is place in the body. Several different commercial preparations are available as kits. These include Tissucol/Tisseel, (Immuno AG, Vienna, now marketed by Baxter), Beriplast P, (Hoechst, West Germany), and Hemaseel (Hemacure Inc. Kirkland, Quebec).

To make a fibrin gel soluble thrombin and fibrinogen are mixed in the presence of calcium 20 chloride. When the components mix, a fibrin adhesive gels is formed because the fibrinogen molecule is cleaved by thrombin to form fibrin monomers. The fibrin monomers spontaneously will polymerize to form a three-dimensional network of fibrin, a reaction that mimics the final common pathway of the clotting cascade, i.e. the conversion of fibrinogen to fibrin sealant. The key to the preparation of commercial preparations is to keep the frinogen and thrombin

components separate until use, so that the polymerization can be controlled with the desired timing before or after application to the body.

Today such use of fibrin as a biologic adhesive has been widely accepted and found application in many fields of surgery. HEMASEEL™ II or Tisseel VH™ are used as an adjunct to hemostasis in surgeries involving cardiopulmonary bypass and treatment of splenic injuries due to blunt or penetrating trauma to the abdomen, when control of bleeding by conventional surgical techniques, including suture, ligature and cautery is ineffective or impractical. The action of these fibrin gels is also used to stop bleeding in surgical procedures involving cardiopulmonary bypass and repair of the spleen. Tisseel VH has also been shown to be an effective sealant as an adjunct in the closure of colostomies.

Collagen gels have been used in tissue culture studies to map gradients of diffusible molecules. The use of collagen gels has permitted the identification and testing of neuronal guidance factors such as netrins (Kennedy, et al. (1994) Cell. 78:425-435). When collagen polymerized it forms a dense protein network. Therefore, like fibrin, it has the potential to act as a tissue adhesive. Moreover, collagen is easy to purify in large quantities.

There are many different types of collagens, and it is a major component of basement membranes in many different body tissues. The form of collagen often used for experimental studies in rodents is type IV collagen because it is easily purified from rat tails.

Not only is collagen a component of the basement membrane in the peripheral nervous system, but it is known that neurons express receptors for collagen. Receptors for collagens are receptors of the integrin class of proteins. One important collagen receptor expressed by neurons is the alpha<sub>1</sub> beta<sub>1</sub> receptor (McKerracher, et al. 1996. Molec. Neurobiol. 12:95-116); this receptor is involved in the promotion of neurite outgrowth. When PC12 cells, a neuronal cell line, are plated on collagen substrates in tissue culture collagen help promote neurite growth in an integrin-dependent fashion. The addition of anti-integrin antibodies block neurite outgrowth. Therefore, the ability of collagen, by itself, has been tested for its ability to promote axon regeneration after spinal cord injury. It was reported that collagen gels by themselves could

foster some axon regeneration after spinal cord injury . . . (Joosten (1995)J. Neurosci. Res. 41:481-490.). However, the observed growth was more of a sprouting response without any long distance regeneration past the glial scar and site of the lesion. In addition, ENBbucollagen has been tested for its ability to promote regeneration after injury in conjunction with the delivery of neurotrophins to injured corticospinal axons . . . (Houweling (1998)Expt. Neurol. 153:49-59). This treatment was not able to support long distance regeneration, although the treated animals had a better sprouting response than the controls. Based on our experiments with fibrin glue (see below), we predict that delivery of C3 in collagen would be improved by the addition of protease inhibitors to prevent lysis of the gel and C3.

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#### SUMMARY OF THE INVENTION

##### **Formulation of C3 plus tissue adhesive**

The invention is the delivery system of a therapeutically active agent (such as for example a Rho antagonist) in a protein matrix that holds the active agent (e.g. Rho antagonist) at the site of application. This delivery system retains the active agent (e.g. Rho antagonist) at the site of CNS injury, allows large doses to be given at the site of injury, and prevents large amounts of the active agent (e.g. Rho antagonist) from leaking into the systemic circulation. The protein matrix can either be based on the fibrin, a protein of the coagulation pathway, or it can be based on collagen, a protein of the extracellular matrix. Both proteins when applied under specific conditions form protein networks when polymerized. These proteins can be applied in soluble form with the additional components necessary for polymerization, together with the Rho antagonist. When the components are mixed immediately before use, polymerization occurs after application to the body site, in our case after application to the CNS.

##### **Kit containing necessary ingredients and instruction for use**

The present invention also relates to a kit suitable for use in the above-described method of delivering fibrin sealant components to a wound site. The kit comprises individually packaged component solutions provided in separate bottles to prevent mixing before use, and an applicator

designed so as to permit mixing of the fibrinogen/Factor XIII and thrombin with C3 at the body site. The kit provides pre-measured amounts of the fibrinogen and factor XIII in one bottle, the thrombin in another bottle, a C3 solution in another bottle. The contents of the bottles would be mixed in a prescribed order, as detailed in the example below. The kit can also include one or 5 more other storage containers which are any necessary reagents including solvents, buffers, calcium chloride, protease inhibitors etc. The kit could be sold as lyophilized or frozen components to preserve the activity of C3 or other Rho antagonist added to the kit.

#### **Therapeutic uses thereof**

- 10 Rho antagonist delivery system may be used in conjunction cell transplantation. Many different cell transplants have been extensively tested for their potential to promote regeneration and repair. These include, but are not restricted to, Schwann cells . . . (Xu, et al. (1996)Exp.Neurol.134:261-272, Guest (1997)Exp. Neurol.148:502-522., Tuszyński, et al. (1998)Cell Transplant.7:187-96), fibroblasts modified to express trophic factors . . . (Liu, et 15 al. (1999)J Neurosci.19:4370-87, Blesch, et al. (1999)J Neurosci.19:3556-66, Tuszyński, et al. (1994)Exp Neurol.126:1-14, Nakahara, et al. (1996)Cell Transplant.5:191-204), fetal spinal cord transplants . . . (Diener and Bregman (1998)J. Neurosci.18:779-793, Bregman (1993)Exp. Neurol.123:2-16), macrophages . . . (Lazarov-Spiegler, et al. (1996)FASEB.J.110:1296-1302), embryonic stem cells . . . (McDonald, et al. (1999)Nat Med.5:1410-2), and olfactory 20 ensheathing glia . . . (Li, et al. (1997)Science.277:2000-2002, Ramon-Cueto, et al. (1998)J Neurosci.18:3803-15, Ramon-Cueto, et al. (2000)Neuron.25:425-35).  
---use in conjunction with neurotrophins

#### **BRIEF DESCRIPTION OF THE FIGURES**

- 25 Figure 1 Schematic diagram of adhesive delivery system of C3 applied to an injured spinal cord.

Figure 2. Diagram to show the model used to show efficacy in vivo. A dorsal hemisection was made in adult mice. Three to four weeks later the anterograde tracer WGA-HRP was injected into the cortex to label the neurons of the corticospinal tract. Two days later the spinal cord was removed and HRP enzymatic activity revealed to detect the CST axons. The corticospinal tract of adult mice was lesion at the T6 level, and the fibrin glue/C3 was added at the time of lesion with a syringe. The expression CST refers to cortical spinal tract.

Figure 3. Longitudinal section of an untreated adult mouse spinal cord 3 weeks after lesion of the corticospinal tract viewed by darkfield microscopy. The fibres were anterogradely labeled from the motor cortex and appear fluorescent. The fibres retract back from the site of the lesion and do not regenerate with treatment.

Figure 4. Use of collagen gels to apply Rho antagonist. A and C are low magnification views of labelled corticospinal axons near the lesion site observed by brightfield; B and D are the respective high magnification views. The lesion can be observed as the area with increased cellularity. (A-B) Collagen implant alone. Axons retract back was observed in untreated controls. (C-D) Collagen plus C3 shows that the axons remain near the lesion, and sprouts into the lesion site are observed.

Figure 5. C3/fibrin treatment stimulates axon regeneration. Top panel: low magnification view observed in darkfield to show the anterogradely-labeled fibres that appear white. Bottom left: high magnification view of the lesion site showing that axons grow through the scar region. High magnification view approximately 7 mm distal to the lesion. Regenerating fibres (arrows) grow long distances.

Figure 6. Results from C3/fibrin treatment from a different animal from that shown in Figure 5. Top panel: Low magnification view of anterogradely labeled axons to show they cross the lesion iste. Bottom panel: regenerating fibres 10 mm from the lesion.

Figure 7. Early functional recovery. Two days after spinal cord injury a control mouse is highly

mobile, but uses its front paws to drag itself forward, and it shows some movement of hindlimb joints. By contrast, a C3-treated animal is able to walk with weight support two days after treatment.

5      Figure 7a Comparison of fibrin, collagen, Gelfoam and Elvax methods of C3 delivery on long-distance regeneration. Animals were treated with the test delivery system without (-C3) or with (+C3) Rho antagonist. Distance of growth of the longest axon was scored by blind examination of at least five sections from each animal. The longest distance of axon growth was scored. Not shown here is that the animals that were not treated with Rho antagonist always showed axon  
10     retraction back from the site of lesion. When axon growth was measured, the distance was measured from the proximal edge of the lesion site. Each point represents data from one animal (approximately 5 sections per animal).

15     Figure 8. Open field test of behavioral recovery. Mice were scored for recovery of function by the 21 point BBB open field test (see experimental section). Two phases of recovery are seen. An early phase, observed in all mice, although the BBB score is higher in the C3-treated mice. The later phase of recovery was only observed after treatment with C3. The C3-treated mice regain almost normal walking behavior.

20     Figure 9. Schematic diagram of a system exploiting a kit in accordance with the present invention.

25     **DETAILED DESCRIPTION**

*Definitions*

The term "fibrin glue" or "fibrin clot" is meant to include any formulations used to make a fibrin clot: eg tisseel VH or see [redacted] Herbert (1998)J. Biomed. Mater. Res.40:551-559, Cheng, et al. (1996)Science.273:510-513, Guest (1997)J. Neurosci. Res.50:888-905). Another definition is any fibrin glue composition not sold as Tisseel, but made by combining fibrinogen, thrombin 5 calcium ions, with or without other components such as factor XIII or apoprotinin.

The term "Rho antagonists" includes, but is not restricted to C3, including C3 chimeric proteins, Y27632l, or other Rho antagonists delivered in the delivery system.

10 The term "Y276321" is defined as a Rho kinase inhibitor that stimulated neurite outgrowth through its ability to inactive the Rho signaling pathway (Uehata, et al. (1997)Nature.389:990-994, Bito (2000)Neuron.26:431-441).

15 The term "nerve injury site" refers to a site of traumatic nerve injury or nerve injury caused by disease. The nerve injury site may be a single nerve (eg sciatic nerve) or a nerve tract comprised of many nerves (eg. damaged region of the spinal cord). The nerve injury site may be in the central nervous system or peripheral nervous system in any region needing repair. The nerve injury site may form as a result of damage caused by stroke. The nerve injury site may be in the brain as a result of surgery, brain tumour removal or therapy following a cancerous lesion. The nerve 20 injury site may result from Parkinson's disease, Alzheimer's disease, Amyotrophic lateral sclerosis, diabetes or any other type of neurodegenerative disease.

#### *Rho GTPase*

25 Rho GTPases include members of the Rho, Rac and Cdc42 family of proteins. Our invention concerns Rho family members of the Rho class. Rho proteins consist of different variants encoded by different genes. For example, PC12 cells express RhoA, RhoB and RhoC (Lehmann et al 1999 IBID). To inactivate Rho proteins inside cells, Rho antagonists of the C3 family type are effective because they inactivate all forms of Rho (eg. RhoA, Rho B etc). In contrast, gene therapy techniques, such as introduction of a dominant negative RhoA family member into a diseased cell, will only inactivate that specific RhoA family member.

*Types of Rho Antagonists**Conventional*

Compounds of the C3 family from *closteridium botulinum* inactive Rho by ADP-ribosylation.

- 5 Recombinant C3 proteins, or C3 proteins that retain the ribosylation activity are also effective in our delivery system and are covered by this invention. In addition, Rho kinase is a well-known target for active Rho, and inactivating Rho kinase has the same effect as inactivating Rho, at least in terms of neurite or axon growth . (Kimura and Schubert (1992)Journal of Cell Biology.116:777-783, Keino-Masu, et al. (1996)Cell.87:175-185, Matsui, et al. (1996)EMBO J.15:2208-2216, Matsui, et al. (1998)J. Cell Biol.140:647-657, Ishizaki (1997)FEBS Lett.404:118-124), the biological activity that concerns this invention. Therefore, chemical compounds such as Y-27632, any other compound are covered by this invention as a preferred delivery in a tissue adhesive system

*Non-Conventional*

- 15 Any compound or molecule that does not have a direct action on Rho itself but works to decrease the function of Rho such as anti-sense oligos to Rho, anti-Rho kinase antibodies, and the like. Such Rho antagonists that can be delivered in a tissue adhesive system are also covered by our invention.
- The C3 polypeptides of the present invention include biologically active fragments and analogs 20 of C3; fragments encompass amino acid sequences having truncations of one or more amino acids , wherein the truncation may originate from the amino terminus, carboxy terminus, or from the interior of the protein. Analogs of the invention involve an insertion or a substitution of one or more amino acids.. Fragments and analogs will have the biological property of C3 that is capable of inactivation Rho GTPases. Also encompassed by the invention are chimeric 25 polypeptides comprising C3 amino acid sequences fused to heterologous amino acid sequences. Said heterologous sequences encompass those which, when formed into a chimera with C3 retain one or more biological or immunological properties of C3. A host cell transformed or transfected with nucleic acids encoding C3 protein or c3 chimeric protein are also encompassed by the invention. Any host cell which produces a polypeptide having at least one of the biological 30 properties of a C3 may be used. Specific examples include bacterial, yeast, plant, insect or

mammalian cells. In addition, C3 protein may be produced in transgenic animals. Transformed or transfected host cells and transgenic animals are obtained using materials and methods that are routinely available to one skilled in the art. Host cells may contain nucleic acid sequences having the full-length gene for C3 protein including a leader sequence and a C-terminal membrane anchor sequence (as shown in FIGS. 1) or, alternatively, may contain nucleic acid sequences lacking one or both of the leader sequence and the C-terminal membrane anchor sequence. In addition, nucleic acid fragments, variants and analogs which encode a polypeptide capable of retaining the biological activity of C3 may also be resident in host expression systems.

10

*Methods of making Rho Antagonists*

The Rho antagonist that is a recombinant proteins can be made according to methods present in the art. The proteins of the present invention may be prepared from bacterial cell extracts, or through the use of recombinant techniques. In general, C3 proteins according to the invention can be produced by transformation (transfection, transduction, or infection) of a host cell with all or part of a C3-encoding DNA fragment in a suitable expression vehicle. Suitable expression vehicles include: plasmids, viral particles, and phage. For insect cells, baculovirus expression vectors are suitable. The entire expression vehicle, or a part thereof, can be integrated into the host cell genome. In some circumstances, it is desirable to employ an inducible expression vector, e.g., the LACSWITCH™ Inducible Expression System (Stratagene, LaJolla, Calif.).

Those skilled in the field of molecular biology will understand that any of a wide variety of expression systems can be used to provide the recombinant protein. The precise host cell used is not critical to the invention. The C3 protein can be produced in a prokaryotic host (e.g., *E. coli* or *B. subtilis*) or in a eukaryotic host (e.g., *Saccharomyces* or *Pichia*; mammalian cells, e.g., COS, NIH 3T3, CHO, BHK, 293, or HeLa cells; or insect cells).

Proteins and polypeptides can also be produced by plant cells. For plant cells viral expression vectors (e.g., cauliflower mosaic virus and tobacco mosaic virus) and plasmid expression vectors

(e.g., Ti plasmid) are suitable. Such cells are available from a wide range of sources (e.g., the American Type Culture Collection, Rockland, Md.). The methods of transformation or transfection and the choice of expression vehicle will depend on the host system selected.

The host cells harbouring the expression vehicle can be cultured in conventional nutrient media adapted as need for activation of a chosen gene, repression of a chosen gene, selection of transformants, or amplification of a chosen gene. One expression system is the mouse 3T3 fibroblast host cell transfected with a pMAMneo expression vector (Clontech, Palo Alto, Calif.). pMAMneo provides an RSV-LTR enhancer linked to a dexamethasone-inducible MMTV-LTR promotor, an SV40 origin of replication which allows replication in mammalian systems, a selectable neomycin gene, and SV40 splicing and polyadenylation sites. DNA encoding a C3 protein would be inserted into the pMAMneo vector in an orientation designed to allow expression. The recombinant C3 protein would be isolated as described below. Other preferable host cells that can be used in conjunction with the pMAMneo expression vehicle include COS cells and CHO cells (ATCC Accession Nos. CRL 1650 and CCL 61, respectively).

C3 polypeptides can be produced as fusion proteins. For example, expression vectors can be used to create lacZ fusion proteins. The pGEX vectors can be used to express foreign polypeptides as fusion proteins with glutathione S-transferase (GST). In general, such fusion proteins are soluble and can be easily purified from lysed cells by adsorption to glutathione-agarose beads followed by elution in the presence of free glutathione. The pGEX vectors are designed to include thrombin or factor Xa protease cleavage sites so that the cloned target gene product can be released from the GST moiety. Another strategy to make fusion proteins is to use the His tag system.

In an insect cell expression system, *Autographa californica* nuclear polyhedrosis virus AcNPV), which grows in *Spodoptera frugiperda* cells, is used as a vector to express foreign genes. A C3 coding sequence can be cloned individually into non-essential regions (for example the polyhedrin gene) of the virus and placed under control of an AcNPV promoter, e.g., the polyhedrin promoter. Successful insertion of a gene encoding a C3 polypeptide or protein will result in inactivation of the polyhedrin gene and production of non-occluded recombinant virus

(i.e., virus lacking the proteinaceous coat encoded by the polyhedrin gene). These recombinant viruses are then used to infect *spodoptera frugiperda* cells in which the inserted gene is expressed (see, Lehmann et al for an example of making recombinant MAG protein).

- 5 In mammalian host cells, a number of viral-based expression systems can be utilised. In cases where an adenovirus is used as an expression vector, the C3 nucleic acid sequence can be ligated to an adenovirus transcription/translation control complex, e.g., the late promoter and tripartite leader sequence. This chimeric gene can then be inserted into the adenovirus genome by *in vitro* or *in vivo* recombination. Insertion into a non-essential region of the viral genome (e.g., region
- 10 E1 or E3) will result in a recombinant virus that is viable and capable of expressing a C3 gene product in infected hosts.

Specific initiation signals may also be required for efficient translation of inserted nucleic acid sequences. These signals include the ATG initiation codon and adjacent sequences. In cases where an entire native C3 gene or cDNA, including its own initiation codon and adjacent

15 sequences, is inserted into the appropriate expression vector, no additional translational control signals may be needed. In other cases, exogenous translational control signals, including, perhaps, the ATG initiation codon, must be provided. Furthermore, the initiation codon must be in phase with the reading frame of the desired coding sequence to ensure translation of the entire insert. These exogenous translational control signals and initiation codons can be of a variety of

20 origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements, transcription terminators

- 25 In addition, a host cell may be chosen which modulates the expression of the inserted sequences, or modifies and processes the gene product in a specific, desired fashion. Such modifications (e.g., glycosylation) and processing (e.g., cleavage) of protein products may be important for the function of the protein. Different host cells have characteristic and specific mechanisms for the post-translational processing and modification of proteins and gene products. Appropriate cell lines or host systems can be chosen to ensure the correct modification and processing of the foreign protein expressed. To this end, eukaryotic host cells that possess the cellular machinery for proper processing of the primary transcript, glycosylation, and phosphorylation of the gene product can be used. Such mammalian host cells include, but are not limited to, CHO, VERO, BHK, HeLa, COS, MDCK, 293, 3T3, WI38, and in particular, choroid plexus cell lines.

- Alternatively, a C3 protein can be produced by a stably-transfected mammalian cell line. A number of vectors suitable for stable transfection of mammalian cells are available to the public; methods for constructing such cell lines are also publicly available. In one example, cDNA encoding the C3 protein can be cloned into an expression vector that includes the dihydrofolate reductase (DHFR) gene. Integration of the plasmid and, therefore, the C3 protein-encoding gene into the host cell chromosome is selected for by including 0.01-300 µM methotrexate in the cell culture medium (as described in Ausubel et al., supra). This dominant selection can be accomplished in most cell types.
- Recombinant protein expression can be increased by DHFR-mediated amplification of the transfected gene. Methods for selecting cell lines bearing gene amplifications are known in the art; such methods generally involve extended culture in medium containing gradually increasing levels of methotrexate. DHFR-containing expression vectors commonly used for this purpose include pCVSEII-DHFR and pAdD26SV(A). Any of the host cells described above or, preferably, a DHFR-deficient CHO cell line (e.g., CHO DHFR cells, ATCC Accession No. CRL 9096) are among the host cells preferred for DHFR selection of a stably-transfected cell line or DHFR-mediated gene amplification.
- A number of other selection systems can be used, including but not limited to the herpes simplex virus thymidine kinase, hypoxanthine-guanine phosphoribosyltransferase, and adenine phosphoribosyltransferase genes can be employed in tk, hgprt, or aprt cells, respectively. In addition, gpt, which confers resistance to mycophenolic acid ; neo, which confers resistance to the aminoglycoside G-418; and hygro, which confers resistance to hygromycin. can be used.
- Alternatively, any fusion protein can be readily purified by utilising an antibody specific for the fusion protein being expressed. For example, a system described in Janknecht et al. (1981) *Proc. Natl. Acad. Sci. USA* 88, 8972, allows for the ready purification of non-denatured fusion proteins expressed in human cell lines. In this system, the gene of interest is subcloned into a vaccinia recombination plasmid such that the gene's open reading frame is translationally fused to an amino-terminal tag consisting of six histidine residues. Extracts from cells infected with recombinant vaccinia virus are loaded onto Ni<sup>2+</sup> nitriloacetic acid-agarose columns, and histidine-tagged proteins are selectively eluted with imidazole-containing buffers.
- Alternatively, C3 or a portion thereof, can be fused to an immunoglobulin Fc domain. Such a fusion protein can be readily purified using a protein A column.
- It is envisioned that small molecule mimetics of the above described antagonists are also encompassed by the invention.
- Method to identify active Rho antagonists**

To test Rho antagonists for activity, a tissue culture bioassay system was used. This bioassay is used to define acitivity of Rho antagonists that will be effective in promoting axon regeneration in spinal cord injury, stroke or neurodegenerative disease.

5      Neurons do not grow neurites on inhibitory myelin substrates. When neurons are placed on inhibitory substrates in tissue culture, they remain rounded. When an effective Rho antagonist is added, the neurons are able to grow neurites on myelin substrates. The time that it takes for neurons to growth neurites upon the addition of a Rho antagonist is the same as if neurons had been plated on growth permissive substrate such as laminin or polylysine, typically 1 to 2 days in cell culture. The results can be scored visually. If needed, a quantitative assessment of neurite growth can be performed. This involved measuring the neurite length in a) control cultures where neurons are plated on myelin substrates and left untreated b) in positive control cultures, such as neurons plated on polylysine c) or treating cultures with different concentrations of the test antagonist.

15     To test C3 in tissue culture, we have found that the best concentration is 25-50 ug/ml. Thus, high concentrations of this Rho antagonist are needed as compared to the growth factors used to stimulate neurite outgrowth. Growth factors, such as nerve growth factor (NGF) are used at concentrations of 1-100 ng/ml in tissue culture. However, growth factors are not able to overcome growth inhibition by myelin. Our tissue culture experiments are all performed in the presence of the growth factor BDNF for retinal ganglion cells, or NGF for PC12 cells.

20     When growth factors have been tested in vivo, typically the highest concentrations possible are used , in the ug/ml range. Also they are often added to the CNS with the use of pumps for prolonged delivery (eg. Ramer et al, IBID). In our in vivo experiments we have used the highest concentrations possible when working with C3 stored as a frozen 1mg/ml solution. The concentration that we have chosen does not prevent the fibrin matrix from polymerizing. We dilute a 1mg/ml solution of C3 with 1/3 volume thrombin and 1/3 vluume fibrinogen solutions (contain calcium and aprotinin). In order to increase the concentration of C3, it would be possible to lyophylize C3 and then resuspend it in the fibrinogen solution. We have tested lyophilized C3 and find it to be active.

25     The Rho antagonist C3 is stable at 37 C for at least 24 hours. We have tested the stability of C3 in tissue culture with the following experiment. The C3 was diluted in tissue culture medium, left in the incubator at 37C for 24 hours, then added to the bioassay system described above, using retinal ganglion cells as the test cell type. These cells were able to extend neurites on inhibitory substrates when treated with C3 stored for 24 hours at 37C. Therefore , the minimum stability is 24 hours. This is in keeping with the stability projection based on amino acid composition (see sequence data, below).

#### *Tissue Adhesive and Formulations used to make them*

30     Different types of tissue adhesive can be made. Examples include collagen gels, fibrin tissue adhesives. Other examples are matrigel <sup>TM</sup>, laminin networks, and adhesives based on a composition of basement membrane proteins that contain collagen.

35     Fibrin sealant has three basic components: fibrinogen concentrate, calcium chloride and thrombin. Other components can be added to affect the time of clot formation, and the size of the

protein network that is formed. Generally when the components mix, a fibrin coagulum is formed in that the fibrinogen molecule is cleaved through the action of thrombin to form fibrin monomers which spontaneously will polymerize to form a three-dimensional network of fibrin, largely kept together by hydrogen bonding. This corresponds to the last phase of the natural blood clotting cascade, the coagulation rate being dependent on the concentration of thrombin used. In order to improve the tensile strength, covalent crosslinking between the fibrin chains is provided for by including Factor XIII in the sealant composition. In the presence of calcium ions, thrombin activates factor XIII to factor XIIIa. Activated factor XIIIa together with thrombin catalyzes the cross-linkage of fibrin and increases the strength of the clot. The strength of the fibrin clot is further improved by the addition of fibronectin to the composition, the fibronectin being crosslinked and bound to the fibrin network formed. During wound healing the clot material undergoes gradual lysis and is completely absorbed. To prevent a too early degradation of the fibrin clot by fibrinolys, the fibrin sealant composition may comprise a plasminogen activator inhibitor or a plasmin inhibitor, such as aprotinin. Such an inhibitor will also reduce the fibrinolytic activity resulting from any residual plasminogen in the fibrinogen composition. Similarly, compositions may include hyaluronic acid (or other polysaccharides), and these may also comprise a hyaluronidase inhibitor such as one or more flavonoids (or corresponding inhibitors for other polysaccharides) in order to prevent degradation (i.e. to prolong the duration) of the hyaluronic acid component by hyaluronidase which is always present in the surrounding tissues. The hyaluronic acid may, as mentioned above, be crosslinked, a commercially available example being Hylan.RTM. (trademark, available from Biomatrix, Ritchfield, N.Y., USA). The hyaluronic acid compositions may e.g. have the form of gels, solutions, etc.

Fibrin clots in any one of the above described embodiments, may be used for the application of a pharmaceutically active substance. By incorporating a drug, such as an antibiotic, a growth factor, etc. into the tissue adhesive it will be enclosed in the fibrin network formed upon application of the tissue adhesive. It will thereby be ensured that the drug is kept at the site of 5 application while being controllably released from the composition.

Fibrin sealant products prepared from human plasma fibrinogen/Factor XIII are available commercially. One product is a tissue glue called Tisseel Fibrin Sealant (Baxter Hyland Immuno Corporation). (Tissucol/Tisseel, Immuno AG, Vienna) and another Beriplast P, Hoechst, West Germany. A frozen formulation of a fibrin glue delivered with a 2 syringe system is Hemaseel 10 made by Hemacure Inc. (Kirkland, Quebec).

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ions, thrombin activates factor XIII to factor XIIIa. Activated factor XIIIa together with thrombin catalyzes the cross-linkage of fibrin and increases the strength of the clot. The strength of the fibrin clot is further improved by the addition of fibronectin to the composition, the fibronectin being crosslinked and bound to the fibrin network formed. During wound healing the 5 clot material undergoes gradual lysis and is completely absorbed. To prevent a too early degradation of the fibrin clot by fibrinolys, the fibrin sealant composition may comprise a plasminogen activator inhibitor or a plasmin inhibitor, such as aprotinin. Such an inhibitor will also reduce the fibrinolytic activity resulting from any residual plasminogen in the fibrinogen composition. Similarly, compositions may include hyaluronic acid 10 (or other polysaccharides), and these may also comprise a hyaluronidase inhibitor such as one or more flavonoids (or corresponding inhibitors for other polysaccharides) in order to prevent degradation (i.e. to prolong the duration) of the hyaluronic acid component by hyaluronidase which is always present in the surrounding tissues. The hyaluronic acid may, as mentioned above, be crosslinked, a commercially available example being Hylan.RTM. (trademark, 15 available from Biomatrix, Ritchfield, N.Y., USA). The hyaluronic acid compositions may e.g. have the form of gels, solutions, etc.

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Germany. A frozen formulation of a fibrin glue delivered with a 2 syringe system is Hemaseel made by Hemacure Inc. (Kirkland, Quebec).

*Making Tissue Adhesive Delivery kits*

In a preferred embodiment, the kit includes the solutions provided in separate bottles to prevent mixing before use, and an applicator designed so as to permit mixing of the fibrinogen/Factor XIII and thrombin with C3 at the body site. The kit would provide pre-measured amounts of the fibrinogen and factor XIII in one bottle, the thrombin in another bottle, a calcium chloride solution in third bottle, and a C3 solution in a forth bottle. The contents of the bottles would be mixed in a prescribed order, as detailed in the example below. The kit can also include one or more other storage containers which are any necessary reagents including solvents, buffers, etc.

The kit could be sold as lyophilized or frozen components to preserve the activity of C3 or other Rho antagonist added to the kit.

The applicator can, for example, take the form of a glass or plastic syringe with disposable needles. With a single syringe system, the components of the kit would be mixed immediately before application to the injury site.

A more elaborate system would allow two syringes to be attached, so that the mixing could take place in the syringe or a mixing compartment of the syringe, before injection. One example of a two syringe system is a Luer lock syringe, such as used for mixing adjuvants.. For this a 3-way stopcock, such as commercially available (Bio-Rad cat #7328103) is attached to the syringe so that the solution can be passed back and forth before attaching the injection needle to the third port of the 3-way stopcock. These are plastic, sterile, and disposable.

Another method of application could be through the use of a clip to hold two syringes, and the clip would have a common plunger to ensure that equal volumes of the thrombin and fibrinogen components are mixed in a chamber with the calcium chloride and C3, before being ejected through the needle.

*Other Ingredients for the Tissue Adhesive Rho Antagonist Delivery System*

Other components can be added to the tissue adhesive to improve efficacy of the treatments.

Such additions include growth factors, protease inhibitors, cytokines, anti-inflammatory compounds, cell transplant systems. Agents that prevent cell death, such as agents that affect the apoptosis pathway could be added components to the delivery system.

*Methods of Packaging Delivery System*

In the preferred formulation, Rho antagonist, fibrinogen and thrombin are mixed together just before application, so that polymerization of the gel occurs in the injured CNS. Therefore, it is important that the fibrinogen and thrombin are package separately. However, the C3 can be packaged separately, or added to either the thrombin or fibrinogen bottles. In another  
5 formulation, the fibrinogen, thrombin and C3 are packaged together, but help at low pH, which prevents polymerization of the gel. Polymerization would be induced by mixing this formulation with a basic component that would neutralize the pH to induce coagulation of the adhesive. In another formulation, the Rho antagonist could be added separately to the fibrinogen/thrombin mix in the form of liposomes or other similar delivery system. Living cells could that secrete C3  
10 could be added as Rho antagonist.

*Method of Applying Rho antagonist in vivo*

Tissue adhesive formulations are typically applied to wound sites with a syringe and needle. The shape of the need determine the type of surface that is formed when the adhesive polymerizes. In some cases, adhesives can be sprayed onto the wound surface, or into the desired region. This  
15 invention covers all types of syringes and needles used to apply fibrin plus Rho antagonists to injured regions of the CNS. In addition, it covers the addition of previously polymerized tissue adhesives with C3 to the wound. For example, fibrin can be polymerized in a teat tube, and forceps used to remove the gel and place it in the body cavity. Similarly, collagen can be applied by pre-polymerization and application by using foceps to place the gel in the injured  
20 spinal cord. One example of this is more fully explained in the example section of this application.

We have tested GelfoamTM, a surgical sponge, and Elvax, a slow release plastic (Lehmann et al 1999, IBID) for the ability to deliver biologically effective concentrations of C3. Neither of these two delivery systems was effective. Therefore, only tissue adhesive formulations have  
25 efficacy in the delivery of C3 to the injured CNS in vivo.

30

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these two delivery systems was effective. Therefore, only tissue adhesive formulations have efficacy in the delivery of C3 to the injured CNS in vivo.

*Therapeutic Applications/Medical Uses*

- 5      The tissue adhesive system for the delivery of Rho antagonists may be useful in many other conditions that affect the central and peripheral nervous system. Treatments that are effective in eliciting sprouting from injured axons are equally effective in treating some types of stroke (Boston life sciences, Sept. 6, 2000 Press Release on-line [www.bostonlifesciences.com](http://www.bostonlifesciences.com)). Since we have shown that our invention elicits sprouting, it is obvious that the treatments can be extended to stroke.
- 10     Similarly, although the subject of this invention is related to delivery of Rho antagonists to the traumatically damaged nervous system, this invention also pertains to damage from neurodegeneration, such as during Parkinson's disease, Alzheimer's disease, prion diseases or other diseases of the CNS were axons are damaged in the CNS environment. In such cases, small volumes of the tissue adhesive with C3 could be injected into the affected region with the use of a syringe.
- 15     The treatment will cause local sprouting to restore function of neurons whose axon processes had retracted in the course of the neurodegeneration.

*Testing the Formulation and Delivery System*

- 20     We tested our formulation in mice after injury of the corticospinal tract. All mice were tested for anatomical regeneration of lesioned axons by anterograde tracing techniques. Some of the mice were also assessed for recovery of locomotion. The details of these experiments are given in the experimental section, the example sections, and the results are shown in the figures.

## EXAMPLES

- 25     *EXAMPLE 1 A kit for a tissue adhesive system.*
- The kit contains:
- 1 vial fibrinogen
- 30     1 vial apropinin solution for reconstitution of fibrinogen
- 1 vial thrombin
- 1 vial calcium chloride solution for reconstitution of thrombin
- 1 vial C3 solution
- 35     1. Lyophilized fibrinogen (75mg/ml) in glycine buffer (2mg/ml NaCl, 4 mg/ml trisodium citrate, 15 mg/ml glycine) was reconstituted in an aprotinin solution 3000 KIU/ml and heated to 37 C. For ease of

handling, a combined heating and stirring device was used  
(appropriate vials contain a maganetic stirrer. This is called solution I.

2. A thrombin solution is prepared. We used lyophilized thrombin  
5 500 IU/ml , 2.4 mg/ml glycine, 8 mg/ml sodium chloride. The  
calcium chloride solution (40 umol CaCl<sub>2</sub>) and thrombin are mixed  
and heated to 37C. This is called solution II.

10 3. A solution of C3 (1 mg/ml) is heated to 37C

4. Equal amounts of solution I, II, and III are mixed, and  
immediately drawn up in a syringe, and added to the injury site where  
polymerization occurs. Thus the C3 is added as part of the fibrin glue  
solution that is placed in the lesion cavity to polymerize.

15

A combined heating and stirring device can be used in conjunction with the kit. For this, small  
magnetic stirrers are included in each of the mixing vials. The vials are then placed in the  
combined mixing and warming device where the magnetic stirrer keeps the solution stirred while  
20 the solution is warming.

Mice that received a dorsal hemisection were treated with the fibrin/C3 adhesive. In some  
experiments, 10  $\mu$ l of 1mg/ml C3 in phosphate buffered saline was added to the lesion site before

applying the C3/fibrin. Behavior recovery was assessed in an open field environment as described by Beattie, Basso and Bresnahan (1995) J. Neurotrauma 12:1-20. Anatomical regeneration was assessed by anterograde labeling of the corticospinal fibres. Three weeks to three months after injury, the corticospinal fibres were labeled by inject the anterograde tracer  
5 WGA-HRP into the motor cortex as described in the art (Huang (1999)Submitted.). Two days later the animals were killed, the spinal cord removed, and longitudinal sections cut and reacted for HRP enzymatic activity, as described ENRfu(Huang (1999)Submitted.). The labeled fibres were observed by microscopy to extend many mm past the lesion site (see figures 5 and  
6) after treatment with C3/fibrin.

10

*EXAMPLE 2. Modification of the kit in example 1.*

The formulation given in example 1 was used with the following modifications. Soultion II is made with the addition of recombinant C3 directly to the solution II vial. In other words,  
15 solution II contains thrombin, calcium chloride and C3. Solution I is loaded in one syringe, solution II is loaded in a second syringe. A syringe clip with a plunger that simultaneously loads both solutions is used. Thus the solutions are mixed as they enter a small chamber before the needle, and the polymerization occurs in situ in the injured region of the CNS where the solution is applied. The system describe here is the Duploject system™.

20

*EXAMPLE 4. Modification of the kit in example 1.*

As example 2, but the C3 solution is mixed in vial I with the fibrinogen. Vial one and vial II are heated and prepared as described in example 1, and injected into the injured CNS with the Duploject system.

5      *EXAMPLE 5 : Collagen gels used as tissue adhesives.*

First collagen is purified. Collagen can be purified from any source, human or mammalian. One source of collagen is the EHS tumor cell line which is passed in mice. We purified collagen from rat tails. The tails were soaked in 70% alcohol for about 20 minutes. The remaining steps were performed under aseptic conditions. The tails are broken about 2 cm from the tip with a hemostat and the tendon is slowly pulled out and placed in a sterile dish. The tendons are cut into small pieces and soaked in acetic acid-water (1:1000) for 48 hours in the cold. 150 ml of solution is used per tail. The solution is centrifuged at 15,000 rpm, 30 min. and stored in aliquots at -10C.

10      hemostat and the tendon is slowly pulled out and placed in a sterile dish. The tendons are cut into small pieces and soaked in acetic acid-water (1:1000) for 48 hours in the cold. 150 ml of solution is used per tail. The solution is centrifuged at 15,000 rpm, 30 min. and stored in aliquots at -10C.

15      Collagen gels with C3 as Rho antagonist are formed in vivo as follows. For treatment of one mouse, 40 µg of C3 was lyophilized. The C3 protein was reconstituted in 10 µl of 7.5% NaHCO<sub>3</sub>. Collagen at 0.7 mg/ml was used, and 25 µl collagen was added to the C3 solution and left at room temperature for 1 hour. A mouse that had received a dorsal hemisection of the spinal cord was treated with 10 µl of 1 mg/ml C3, then the collagen containing C3 was added as a polymerized clot to the lesion site. Anatomical regeneration of transected cortical spinal fibres was assessed as described in the detailed description of the invention.

20      polymerized clot to the lesion site. Anatomical regeneration of transected cortical spinal fibres was assessed as described in the detailed description of the invention.

*EXAMPLE 6. Procedure to make recombinant C3 as a Rho antagonist.* We made recombinant C3 protein as follows. The plasmid pGEX2T-C3 coding for the glutathione-S-transferase (GST)-C3 fusion protein was obtained from N. Lamarche (McGill Univ.). Bacteria were transformed with pGEX2T-C3, allowed to grow overnight, and sonicated to break open the cells. The recombinant protein was purified by affinity chromatography as described<sup>ENRfu</sup>(Ridley and Hall (1992)Cell.70:389-399). The GST fusion protein was cleaved by thrombin, and thrombin was removed by incubation with 100µl of p-aminobenzamidine agarose-beads (Sigma). The C3 solution was dialyzed against PBS, and sterilized with a 0.22 µm filter. The C3 concentration was evaluated by protein assay (DC assay, BioRad Labs, Missassauga, Ont.) and C3 purity was controlled by SDS-PAGE analysis.

*EXAMPLE 7: Testing the Fibrin-Rho-Antagonist Formulation using the Delivery System*

To test the tissue adhesive system a rodent model of spinal cord injury was used. For this, Balb-c mice were anaesthetized with 0.6 ml/kg hypnorm, 2.5 mg/kg diazepam and 35 mg/kg ketamine. A section of the thoracic spinal cord was exposed using fine rongers to remove the bone. A dorsal hemisection was made to cut the dorsal columns at level T6. The fibrin/C3 adhesive was injected immediately after injury. As control another group of animals received fibrin alone, and a third group received no treatment. The following day behavioural testing began, and continued for three weeks. The animals were placed in an open field environment that consisted of a rubber mat approximately 4' X3' in size. The animals were left to move randomly, the movement of

the animals were videotaped. For each test two observers scored the animals for ability to move ankle, knew and hip joints in the early phase of recovery. In the intermediate phase, the ability to support weight and correct placement of the feet was assessed (dorsal or plantar placement). In the late phase of recovery, the animals were assessed for correct foot position, trunk stability ,  
5 and foot drag. Only animals that received C3/fibrin reached the late phase of recovery. Untreated control animals did not typically pass beyond the early phase of recovery.

### **Experimental section**

#### **Spinal cord injury**

- 10 To study The CST was cut bilaterally by a dorsal hemisection extending past the central canal (1 (Fig. 2) at the T6 level. Balb-c mice were anaesthetized with 0.6 ml/kg hypnorm, 2.5 mg/kg diazepam and 35 mg/kg ketamine. A section of the thoracic spinal cord was exposed using fine rongers to remove the bone, and a dorsal hemisection was made at level T6. Fine sissors were used to cut the dorsal half of the spinal cord, and it was recut a second time with fine knife to  
15 ensure all lesions extended past the central canal. Three weeks to four weeks after injury, the corticospinal fibres were labeled by injection the anterograde tracer WGA-HRP into the motor cortex as into 6 sites. For injection into the motor cortex a pulled glass pipette was used . Two days later the animals were perfused transcardially with saline then 4% paraformaldehyde and the spinal cords and brains were removed.  
20 C3 toxin was delivered locally to the site of the lesion by a fibrin-based tissue adhesive delivery system (Figure 1). Recombinant C3 was mixed with fibrinogen and thrombin in the presence of CaCl<sub>2</sub>. Fibrinogen is cleaved by thrombin, and the resulting fibrin monomers polymerize into a three-dimensional matrix. We added C3 as part of a fibrin adhesive, which polymerized within about 10 seconds after being placed in the injured spinal cord. Anterograde tracing with WGA-HRP was  
25 used to study anatomical regeneration past the site of lesion in three groups of animals: animals treated with fibrin plus C3 (C3/fibrin), animals treated with fibrin alone, and animals that did not received treatment after injury (see figure 7). With no treatment, transected CST axons retract back

from the site of lesion from 500 um to 1 mm (Fig. 3). Animals treated with fibrin alone showed less axon retraction, and sprouting of axons was observed to extend towards the scar . Application of C3 to the injured spinal cord elicited an extensive sprouting of CST axons into the dorsal white matter, and the axons grew into the scar and extended past the lesion (Fig. 4). A long distance regeneration of individual CST axons and axon bundles was elicited by C3 (Fig. 5), but not in untreated or fibrin controls. This regeneration was significantly different from any growth observed following treatment with fibrin alone (quantitative data not finished).

We also tested several different tissue adhesive delivery systems. When C3 was delivered in collagen gels less axon retraction was observed, but the same extent of axon regeneration was not observed as with fibrin. GelfoamTM , a surgical sponge, was also tested. Gelfoam was not as effective as fibrin as promoting long-distance regeneration (Fig. 7). We also tested a non-biological material, Elvax, which is a polymer-based artificial release system (see Lehmann et al, 1999 IBID). This system was not effective in allowing cut axons access to C3.

To test functional recovery following treatment of injured spinal cord with C3, three groups of animals were score for locomotor behaviour in an open field environment according to the 21 point BBB scale (Basso et al. ). The animals were examined by two reviewers and were placed alone in an open field environment that consisted of a rubber mat approximately 4' X 3' in size. Each animal was videotaped for approx. 3 min. For the early and intermediate phases, the BBB scores were derived following observation, and confirmed by video analysis. In the late phase of recovery, the BBB score was determined from the videos projected on a computer at  $\frac{1}{4}$  speed from sequences of 4 steps or more. The BBB test includes three phases of recovery: an early phase (scores 1-7) of joint movement, an intermediate phase (score 8-13) where weight support and foot placement (dorsal or plantar) are assessed, and a late phase of coordinated movements (scores 14-21) where correct foot position, and foot drag are examined. The C3 treated animals rapidly regained the ability to support weight (Fig. 9 ) while control animals moved mostly by the action of their forelimbs. The control groups entered the intermediate recovery phase with the ability to support weight within one weeks, at which point they obtained their recovery plateau. Animals that received C3 treatment continues to recover over the 1 month period of observation, and recovered coordinated movement and almost normal stepping (Fig. 8).

In rats that receive a contusion injury the recovery period depends on the severity and location of the lesion . Typically, rats reach a plateau of recovery by about two week, whereas after dorsal hemisection in mice we find the plateau of recovery is reached within about 1 week. The remarkable improvement in C3-treated mice within one day of spinal cord lesion is likely due to changes in the local spinal cord circuitry. These local changes might result from the robust sprouting immediately after application of C3 is applied to the transected axons. Rates of axon growth *in vivo* are known to be approximately the same as the slow axonal transport rate of 50-200 um/hr. It is also possible that the local effects on the spinal cord are mechanistically different by acting on central pattern generators implicated in walking behaviors. Most importantly, treated mice performed better immediately after lesion, and they recovered almost normal walking patterns by one month (Figure 8). We attribute this slower phase of recovery to the long-distance regeneration of axons that was induced by C3 (Figure 4). Moreover, while we only flowed the CST axons in this study, our treatments also are likely stimulate growth from other transected axonal populations.

15

#### Production of recombinant C3

C3 is a protein product made by the bacteria *Clostridium botulinum*. The fragment containing the C3 gene was cloned into a pGEX vector (Pharmacia), now referred to as pGEX2T-C3, and this vector was obtained from Nathalie Lamarche of McGill University. To confirm the C3 sequence corresponded to that reported in the literature we had the insert sequenced at a commercial sequencing facility (see sequence below). The C3- containing pGEX vector was transformed into the RR1 strain of *E. coli* (GIBCO). Bacteria were grown in L-Broth (10g/L Bacto-Tryptone, 5g/L Yeast Extract, 10g/L NaCl (Fisher Scientific) with Ampicillin(BMC-Roche) at 50ug/ml in a shaking incubator for 1 hr at 37°C. Isopropyl β-D-thiogalactopyranoside(IPTG), ( GIBCO) was added to a final concentration of 0.5mM to induce production of recombinant protein and the culture was grown for a further 6 hrs at 37°C. Bacterial pellets were obtained by centrifugation, in 250ml centrifuge bottles, at 6000rpm at 4C for 5min. Pellets can be kept frozen at -80°C at this time.

30 5mls of Buffer A(50mM Tris, pH7.5, 50mM NaCl, 5mM MgCl<sub>2</sub>, 1mM DTT) + 1mM PMSF was added to each pellet. Pellets were resuspended and transferred to a 50ml plastic

beaker on ice, and a further 5 mls of buffer A was used to wash the centrifuge bottles. Total volume of buffer A + pellets from a 2 L culture is usually 30-40mls. The pellets, on ice, were sonicated 5 X 30 secs using a BRANSON SONIFIER 450 probe sonicator. Bacteria were cooled on ice 1 minute between sonications. The sonicate was centrifuged in a Sorvall SS-34 rotor at 5 10,000 rpm for 10 min at 4°C to clarify supernatant.

Glutathione-agarose beads (SIGMA#G-4510) were purchased as a lyophilized powder and the beads were swollen in deionized water, then stored in 1M NaCl at 4°C. Five ml of the beads (50%v/v) were washed in a 50ml tube filled with buffer A (no PMSF). Tube was centrifuged at 2000 rpm (500g) for 5min, water was removed, and replaced with buffer A.  
10 These beads were added to the cleared bacterial supernatant, and mixed for 1-2hrs at 4°C. The beads were washed 4 times with buffer B (buffer A, NaCl is 150mM, no PMSF), then 2X with buffer C (buffer B+2.5mM CaCl<sub>2</sub>). Washes were poured out and the beads retained each time. Next, 5mls of thrombin(Bovine, Plasminogen-free, CALBIOCHEM #605160) 20U at (50%v/v )  
15 was added to the beads to cleave the C3 from the GST affinity purification tag (see cleavage site in the nucleotide sequence given below). This reaction s was left overnight, with mixing, at 4°C.

The beads are loaded into an empty 10 ml column, PBS (phosphate buffered saline) was added to the column and 20 1 ml aliquots were collected. To determine the location of the protein peak, 0.5ul spots wer put on a nitrocellulose sheet, from each aliquot, and this is stained with Amido Black(Bio-rad) as a protein dot-blot. Aliquots containing C3 were pooled and 20 20 uls p-Aminobenzamidine (SIGMA#A7155) was added. The solution was mixed for 30min at 4°C to remove the thrombin. The C3 is centrifuged to remove the p-aminobenzamidine, and then concentrated using a CENTRIPREP-10 concentrator (AMICON). The concentrated C3 is then passed through a PD-10 column (PHARMACIA, containing Sephadex G-25M) and 10 0.5 ml aliquots are collected. A dot-blot is done on these aliquots, and the appropriate aliquots  
25 (usually 3 tubes) are pooled (total volume about 1.5 mls). The purified recombinant protein was filter-sterilized, aliquoted, and stored at -80C. A protein assay was done on a small amount to determine precisely the concentration. Purity of the recombinant C3 was evaluated by SDS polyacrylamide gel electrophoresis. Bioactivity was assed with a bioassay using either retinal ganglion cells or PC12 cells (see ientification of Rho antagonist section).

**SEQUENCE of Rho antagonist C3 used in the experiments**

Nucleotide sequence including part of the plasmid GST sequence. Arrow show thrombin cleavage site. Underlined sequence shows additional coding sequence translated in our recombinant protein that is not reported in the literature.

Both strands were sequences to verify that there were no errors in the sequence.

↓

5' GTG GCG ACC CTT CCC AAA TCG GAT CTG GTT CCG CGT GGA TCC TCT  
 10 AGA GTC GAC CTG CAG GCA TGC AAT GCT TAT TCC ATT AAT CAA AAG GCT  
 TAT TCA AAT ACT TAC CAG GAG TTT ACT AAT ATT GAT CAA GCA AAA GCT TGG  
 GGT AAT GCT CAG TAT AAA AAG TAT GGA CTA AGC AAA TCA GAA AAA GAA  
 GCT ATA GTA TCA TAT ACT AAA AGC GCT AGT GAA ATA AAT GGA AAG CTA  
 15 AGA CAA AAT AAG GGA GTT ATC AAT GGA TTT CCT TCA AAT TTA ATA AAA CAA  
 GTT GAA CTT TTA GAT AAA TCT TTT AAT AAA ATG AAG ACC CCT GAA AAT ATT  
 ATG TTA TTT ANA GGC GAC GAC CCT GCT TAT TTA GGA ACA GAA TTT CAA  
 AAC ACT CTT CTT AAT TCA AAT GGT ACA ATT AAT AAA ACG GCT TTT GAA AAG  
 GCT AAA GCT AAG TTT TTA AAT ANA GAT AGA CTT GAA TAT GGA TAT ATT AGT  
 20 ACT TCA TTA ATG AAT GTT TCT CAA TTT GCA GGA AGA CCA ATT ATT ACA AAA  
 TTT AAA GTA GCA AAA GGC TCA AAG GCA GGA TAT ATT GAC CCT ATT AGT  
 GCT TTT CAG GGA CAA CTT GAA ATG TTG CTT CCT AGA CAT AGT ACT TAT  
 CAT ATA GAC GAT ATG AGA TTG TCT TCT GAT GGT AAA CAA ATA ATA ATT  
 ACA GCA ACA ATG ATG GGC ACA GCT ATC AAT CCT AAA TAA 3'

25 Nucleotide sequence of recombinant C3 protein:

1	GGATCCCTCTA GAGTCGACCT GCAGGGCATGC AATGCTTATT CCATTAATCA
51	AAAGGCTTAT TCAAATACTT ACCAGGGAGTT TACTAATATT GATCAAGCAA
101	AAGCTTGGGG TAATGCTCAG TATAAAAAGT ATGGACTAAG CAAATCAGAA
151	AAAGAAGCTA TAGTATCATA TACTAAAAGC GCTAGTGAAA TAAATGGAAA
201	GCTAAGACAA AATAAGGGAG TTATCAATGG ATTTCCCTCA AATTTAATAA
251	AACAAGTTGA ACTTTTAGAT AAATCTTTA ATAAAATGAA GACCCCTGAA
301	AATATTATGT TATTANAGG CGACGACCT GCTTATTTAG GAACAGAATT
351	TCAAAACACT CTTCTTAATT CAAATGGTAC AATTAATAAA ACGGCTTTG
401	AAAAGGCTAA AGCTAAGTTT TTAAATANAG ATAGACTTGA ATATGGATAT
451	ATTAGTACTT CATTAATGAA TGTTCCTCAA TTTGCAGGAA GACCAATTAT
501	TACAAAATTT AAAGTAGCAA AAGGCTCAA GGCAGGGATAT ATTGACCCTA
551	TTAGTGCCTT TCAGGGACAA CTTGAAATGT TGCTTCCTAG ACATAGTACT
601	TATCATATAG ACGATATGAG ATTGTCTTCT GATGGTAAAC AAATAATAAT
651	TACAGCAACA ATGATGGCA CAGCTATCAA TCCTAAATAAT

Amino acid sequence (one letter code)

Amino acids in bold highlight differences from published sequence (Popoff et al. (1990) Nucl. Acid. Res. 18:1291. EMBL accession no. X511464.) The 11 N-terminal sequences are additional; there is a single amino acid change of an alanine (hydrophobic) to glutamic acid (Q).

5

**GSSRVDLQAC** NAYSINQKAY SNTYQEFTNI DQAKAWGNAQ YKKYGLSKSE KEAIVSYTKS  
ASEINGKLRQ NKGVINGFPS NLIKQVELLD KSFNKMKTPE NIMLFXGDDP AYLGTEFQNT  
LLNSNGTINK TAFEKAKAKF LNXDRLEYGY ISTSLMNVSQ FAGRPIITKF KVAKGSKAGY  
IDPISAFQQQ LEMLLPRHST YHIDDMRLSS DGKQIIITAT MMGTAINPK

10

Number of amino acids: 229

Molecular weight: 25507.5

5 Theoretical pI: 9.43

Amino acid composition:

10	Ala (A)	18	7.9%
	Arg (R)	6	2.6%
	Asn (N)	18	7.9%
	Asp (D)	10	4.4%
	Cys (C)	1	0.4%
15	Gln (Q)	12	5.2%
	Glu (E)	10	4.4%
	Gly (G)	16	7.0%
	His (H)	2	0.9%
	Ile (I)	18	7.9%
20	Leu (L)	17	7.4%
	Lys (K)	23	10.0%
	Met (M)	7	3.1%
	Phe (F)	10	4.4%
	Pro (P)	7	3.1%
25	Ser (S)	20	8.7%
	Thr (T)	14	6.1%
	Trp (W)	1	0.4%
	Tyr (Y)	11	4.8%
	Val (V)	6	2.6%
30	Asx (B)	0	0.0%
	Glx (Z)	0	0.0%
	Xaa (X)	2	0.9%

35 Total number of negatively charged residues (Asp + Glu): 20  
 Total number of positively charged residues (Arg + Lys): 29

Estimated half-life:

40

The N-terminal of the sequence considered is G (Gly).

45 The estimated half-life is: 30 hours (mammalian reticulocytes, in vitro).  
 >20 hours (yeast, in vivo).  
 >10 hours (Escherichia coli, in vivo).

Instability index:

50

The instability index (II) is computed to be 26.88  
 This classifies the protein as stable.

Aliphatic index: 75.07

Grand average of hydropathicity (GRAVY): -0.479

5

As discussed herein in accordance with the present invention a therapeutically active agent for facilitating axon growth may be delivered (in a flowable matrix forming substance) to a (nerve) lesion site, for example, by injection using known syringe type glue or sealant devices modified as necessary or desired (e.g. by addition of a further substance container); examples of known delivery devices, systems, mechanisms, matrix forming compositions, and teh like are shown for example in U.S. patent no. 5,989215, U.S. patent no. 4,978,336, U.S. patent no. 4,631,055, U.S. Pat. No. 4,359,049, U.S. Pat. No. 4,974,368, U.S. patent no. 6,121,422, U.S. patent no. 6,047,861, U.S. patent no. 6,036,955, U.S. patent no. 5,945,1115, U.S. patent no. 5,900,408 , U.S. patent no. 6,124,273, U.S. patent no. 5,922,356, and in particular U.S. patent no. 6,117,425; the entire contents of each of these patents is incorporated herein by reference.

A sufficient amount of a therapeutically active agent for facilitating axon growth may be dispersed in a stable flowable (known) type of (proteinaceous) matrix forming material. Once delivered to the desired lesion site the resulting in situ or in vivo matrix (e.g. gel or crosslinked substances) inhibits the migration or diffusion of the agent from the site of injection, so as to maintain the primary effect of the agent in the region of injection, i.e. in the area of the lesion. In any event the active agent is to be present in an amount effective to facilitate axon growth.

A substantially uniform dispersion of the active agent may be initially be formed so as to provide a concentrated amount of active agent in a physiologically acceptable matrix forming material . The matrix forming material may be comprised of any (known) individual or combination of peptides, proteins etc. which provides for stable placement, or combinations thereof. Of particular interest is a collagen material, a fibrinogen material, or derivatives thereof; other high molecular weight physiologically acceptable biodegradable protein matrix forming materials may if desired be used. The active agent may, for example, be incorporated in as sufficient concentration so as to provide the desired or effect the desired sustained release.

5       Typically when estimating doses in different animal species, the same weight ration is used. We apply 40 ug protein per 20 gm mouse. Therefore, we anticipate that the ideal dose should be approximately 3 gm per 60 kg person. We expect that the dose necessary will depend on the size of the lesion and the time of application (acute or chronic) spinal cord injury. In cases of chronic injury, there is often a necrotic center in the spinal cord, and higher doses may be required.

10      The matrix forming material may be a one-component adhesive or sealant type material (e.g. collagen material); alternatively it may be a mult-component adhesive or sealant (e.g. a fibrinogen based material). The matrix may be a human protein matrix or if necessary or desired a non-human protein matrix; preferably a human protein matrix.

15      The (proteinaceous) matrix forming material is flowable for injection, but once in vivo it provides for stable placement, of the active agent in the lesion area; i.e. after injection, the active agent is released into the immediate environment the matrix providing a medium for prolonged contact between a lesion site and the active agent (i.e. axon growth facilitator or stimulant).

20      The matrix forming material(s) is (are) of course to be chosen on the basis that the materials and resultant formed matrix will be capable on the one hand of holding the active agent for release in situ and on the other without preventing the therapeutic effect thereof, i.e. the matrix is to be therapeutically acceptable. The choice of active agent may be determined empirically through appropriate or suitable assays keeping in mind that the matrix etc. are to be therapeutically acceptable.

25      The present invention in an aspect relates to a biocompatible, supplemented tissue sealant or adhesive composition comprising:

30      (i) at least one supplement selected from the group consisting of therapeutically active agents for facilitating axon growth; and

35      (ii) a flowable carrier component capable of forming a pharmaceutically or therapeutically acceptable matrix (in vivo);

40      wherein said supplement is releasable from said matrix into the adjacent external environment

(e.g. for a sustained period of time).

- 5 By way of example only in accordance with the present invention a method of applying an supplemented solution of polymerizable fibrin to a desired lesion site, may comprise
  - a) affixing a cartridge containing immobilized thrombin to a syringe containing a solution of fibrinogen,
  - 10 b) contacting the solution of fibrinogen with immobilized thrombin under conditions resulting in an activated solution of polymerizable fibrin by passing the solution of fibrinogen through the cartridge containing immobilized thrombin,
  - c) adding to the fibrinogen solution or to the activated solution a supplement
    - (i) at least one supplement selected from the group consisting of therapeutically active agents for facilitating axon growth; and
    - 15 c) delivering the supplemented activated solution of polymerizable fibrin to the desired lesion site (e.g. a central nervous system (CNS) lesion site or a peripheral nervous system (PNS) lesion site) under conditions which result in polymerized fibrin at the lesion site having dispersed therein the supplement  
wherein said supplement is released from said fibrin matrix into the adjacent external environment..
- 20
- 25

In accordance with another aspect the present invention relates to a kit comprising, in suitable container means (e.g. separate means):

- 30 (a) a first pharmaceutical composition or substance comprising a biological agent capable of facilitating axon growth; and

5                   (b) a second pharmaceutically or therapeutically acceptable component comprising a single flowable carrier component or two or more separate components capable once intermingled of forming a flowable carrier component, said flowable carrier components each being capable of forming a pharmaceutically or therapeutically acceptable matrix (e.g. proteinaceous matrix, i.e. a proteinaceous glue, proteinaceous sealant, proteinaceous gel, etc.; e.g. a human derived proteinaceous matrix) in vivo at a (nerve) lesion site.

In particular the present invention provides a (axon growth stimulation) kit comprising

10                  a) a first container means (e.g. one or more separate containers) for containing a flowable carrier component(s) or two or more separate components capable once intermingled of forming a flowable carrier component, said flowable carrier components each being capable of forming a pharmaceutically or therapeutically acceptable matrix (e.g. proteinaceous matrix, i.e. a proteinaceous glue, proteinaceous sealant, proteinaceous gel, etc.; ie.g. a human derived proteinaceous matrix) in vivo at a (nerve) lesion site (e.g. a central nervous system (CNS) lesion site or a peripheral nervous system (PNS) lesion site) and

15                  b) a second container means for containing a therapeutically active agent for facilitating axon growth at the lesion site

20                  wherein said therapeutically active agent supplement is releasable from said in vivo matrix into the adjacent external environment (e.g. for a sustained period of time). Alternatively, if desired or as necessary, the first and second container means may be the same, (e.g. a container may hold collagen an C3). The kit may if desired or necessary additionally comprise means for dispersing (i.e. co-mingle, blend, etc.) the therapeutically active agent in said flowable carrier component so as to form a flowable axon growth stimulation composition as well as means for delivering the flowable axon growth stimulation composition to the lesion site (e.g. syringe needle). The pharmaceutically acceptable matrix may as discussed herein be a collagen matrix or a fibrin matrix.

25                  In accordance with the present invention the therapeutically active agent for facilitating axon growth may for example be a Rho antagonist which may be identified by an assay method comprising the following steps:

30                  a) culturing neurons on inhibitory substrate or a substrate that incorporates a growth-inhibitory protein.

b) Exposing the cultured neuron of step a) to a candidate Rho antagonist in an amount and for a period sufficient to permit growth of neurites , and determining if the candidate has elicited neurite growth from the cultured neurons of step a), the appearance of neurites being suggestive or indicative of a Rho antagonist.

5

A compound can be confirmed as a Rho antagonist in one of the following ways:

a) Cells are cultured on a growth inhibitory substrate as above, and exposed to the candidate Rho antagonist.

10 b) Cells of step a) are homogenized and a pull-down assay is performed. This assay is based on the capability of GST-Rhotektin to bind to GTP-bound Rho. Recombinant GST-Rhotektin or GST rhotektin binding domain (GST-RBD) is added to the cell homogenate made from cells cultured as in a). We have found that inhibitory substrates activate Rho, and that this activated Rho is pulled down by (GST-RBD). Rho antagonists will block activation of Rho, and therefore, and effective Rho antagonist will block the detection of Rho when cell are cultured as described  
15 by a) above.

c) An alternate method for this pull-down assay would be to use the GTPase activating protein, Rho-GAP as bait in the assay to pull down activated Rho, as described (Diekmann and Hall, 1995. In Methods in Enzymology Vol. 256 part B 207-215).

20 Another method to confirm that a compound is a Rho antagonist is as follows:

When added to living cells antagonists that inactivate Rho by ADP-ribosylation of the effector domain can be identified by detecting a molecular weight shift in Rho (Lehmann et al, 1999 Ibid). The molecular weight shift can be detected after treatment of cells with Rho antagonist by homogenizing the cells, separating the proteins in the cellular homogenate by SDS  
25 polyacrylamide gel electrophoresis. The proteins are transferred to nitrocellulose paper, then Rho is detected with Rho-specific antibodies by a Western blotting technique.

Another method to confirm that compound is a Rho-kinase antagonist is as follows:

a) Recombinant Rho kinase tagged with myc epitope tag, or a GST tag is expressed in HeLa cells or another suitable cell type by transfection.

5 b) The kinase is purified from cell homogenates by immunoprecipitation using antibodies directed against the myc tag or the GST tag.

c) The recovered immunoprecipitates from b) are incubated with [<sup>32</sup>P] ATP and histone type 2 as a substrate in the presence or absence of the Rho kinase antagonist. In the absence of Rho kinase activity the Rho kinase antigen is able to block the phosphorylation activity of Rho

10 kinase, and as such identified the compound as a Rho kinase antagonist.

15

Figure 9 illustrates in schematic fashion a system exploiting a kit of the present invention for mixing and delivering a supplemented matrix forming material. An actual apparatus may for example be of multi-cartridge syringe type as known or modified as necessary or desired.

20 The kit portion of the illustrated system comprises a container means 1 for fibrinogen material, a container means 2 for thrombin material and a container means 4 for a therapeutically active agent for facilitating axon growth (e.g. C3 or a modified or hybrid C3). If desired or necessary the kit portion may include additional containers for the separate containment of other desired or necessary components; as shown the system in figure includes in dotted outline an additional container means for the flowable matrix forming part of the kit. The system also includes a mixing container 6 wherein the C3 (hybrid) is mixed with the matrix forming elements to form the supplemented flowable matrix forming carrier. The feed line 8 is

indicative of the addition of C3 to the container 8 whereas the feed ligne 10 is indicative of the addition of the flowable matrix forming elements from containers 1 and 2 and which is formed from the merging of feed lines 12 and 13. The mixing in the container means 6 may be effected or carried out in any suitable (known) fashion, (e.g. simple stirring with a magnetic stirrer. The output line 15 of the mixing container is indicative of the delivery of the supplemented mixture to the lesion site (e.g. by needle ( e.g. syringe), pipette, etc.

5  
Although in figure 9 the therapeutically active agent for facilitating axon growth (e.g. C3) is shown As being associated with a separate container 4, if so desired or as necessary the  
therapeutically active agent may be associated with a container holding a flowable carrier  
10 component (e.g. a container may hold fibrinogen and C3)..

15

I claim:

1. A (axon growth stimulation) kit comprising

5           a first container means (e.g. one or more separate containers) for containing a flowable carrier component or two or more separate components capable once intermingled of forming a flowable carrier component, said flowable carrier components each being capable of forming a therapeutically acceptable matrix (e.g. proteinaceous matrix, i.e. a proteinaceous glue, proteinaceous sealant, proteinaceous gel, etc.; e.g. a human derived proteinaceous matrix) in vivo at a (nerve) lesion site (e.g. a central nervous system (CNS) lesion site or a peripheral nervous system (PNS) lesion site) and

10           a second container means for containing a therapeutically active agent for facilitating axon growth at the lesion site

15           wherein said therapeutically active agent is releasable from said in vivo matrix into the adjacent external environment (e.g. for a sustained period of time).

20           2. A (axon growth stimulation) kit as defined in claim 1 comprising means for dispersing (i.e. co-mingle, blend, etc.) the therapeutically active agent in said flowable carrier component so as to form a flowable axon growth stimulation composition and

25           means for delivering the flowable axon growth stimulation composition to the lesion site.

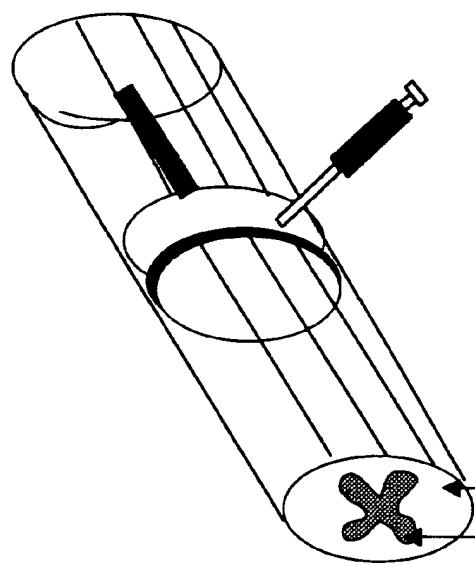
25           3. A (axon growth stimulation) kit as defined in claim 1 wherein said pharmaceutically acceptable matrix is a collagen matrix.

30           4. A (axon growth stimulation) kit as defined in claim 1 wherein said pharmaceutically acceptable matrix is a fibrin matrix.

Figure 1

## Delivery of Rho-antagonist tissue adhesive formulation.

1. Application of tissue adhesive + Rho antagonist to the injured spinal cord with a pipette.



2. Axon regeneration through the adhesion matrix + C3

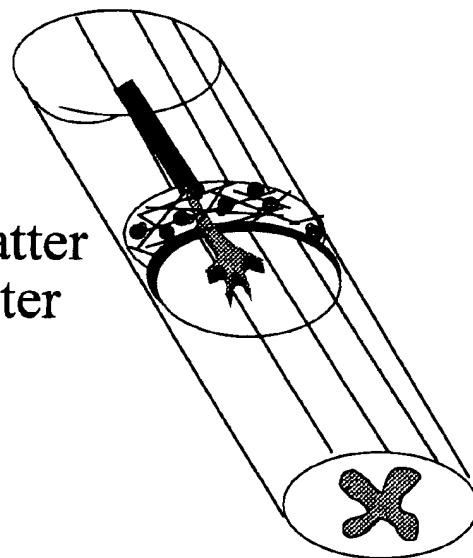


Figure 2

## Lesion of Corticospinal tract

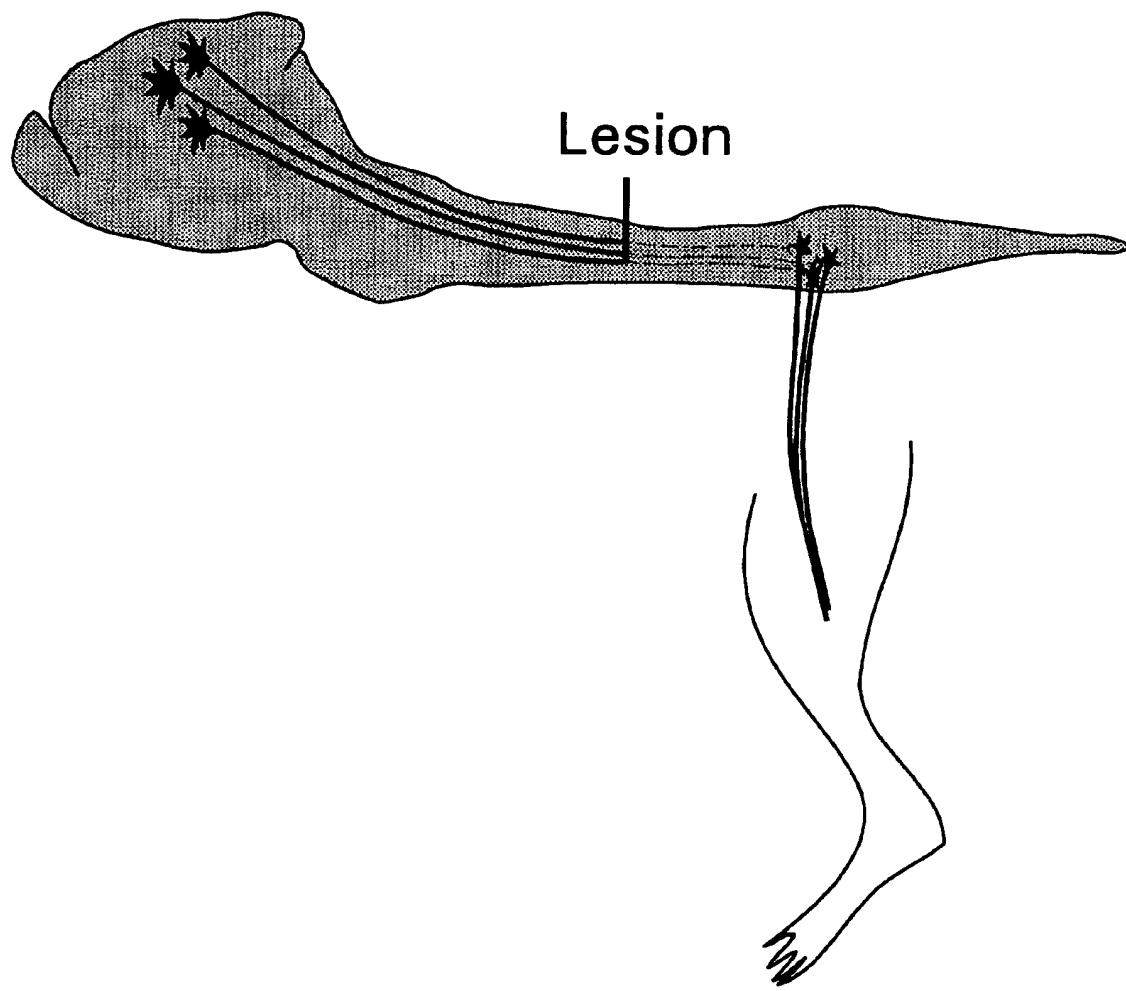
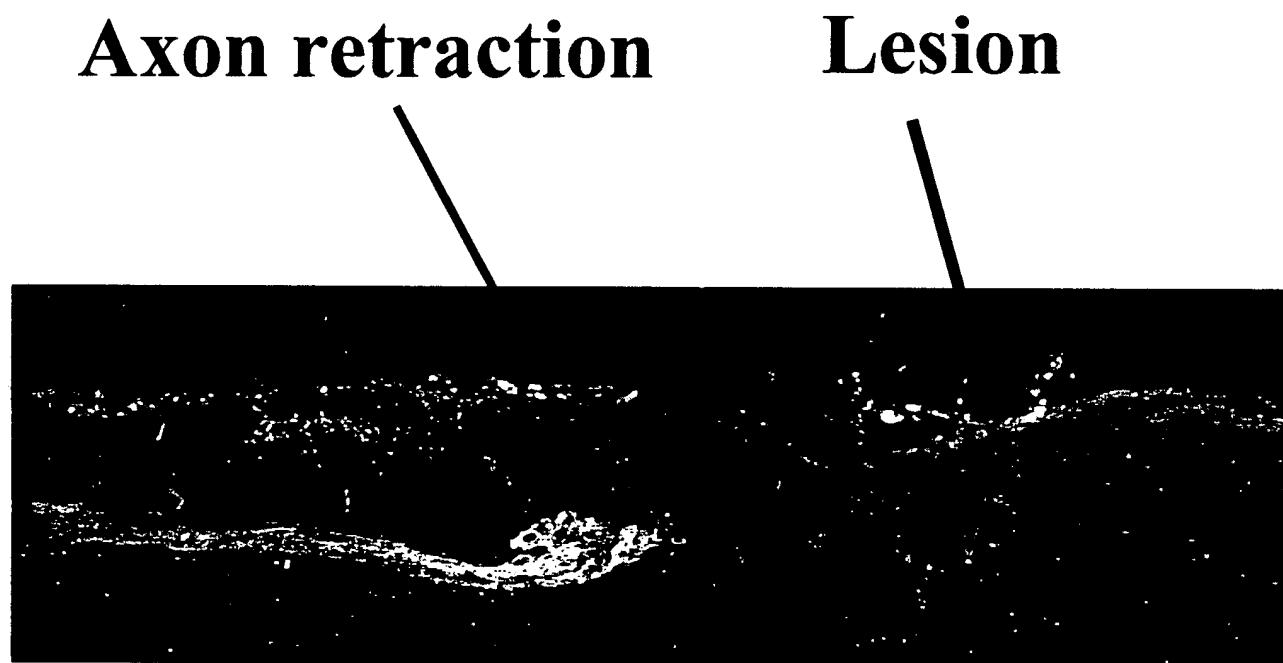


Figure 3

**Corticospinal tract lesion  
(untreated adult mice)**



**Figure 4**

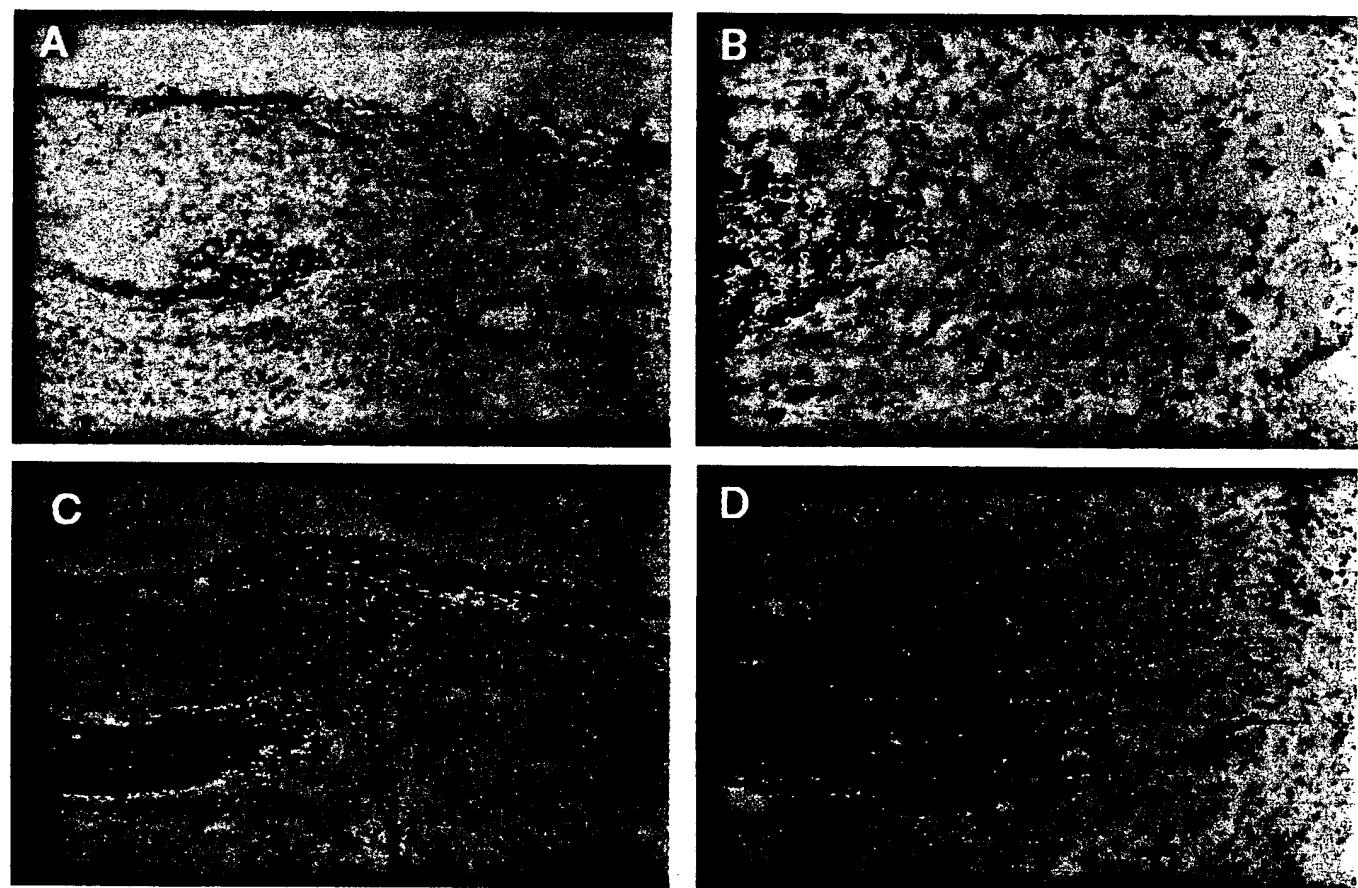


Figure 5

**Effect of C3/fibrin treatment on injured corticospinal tract**

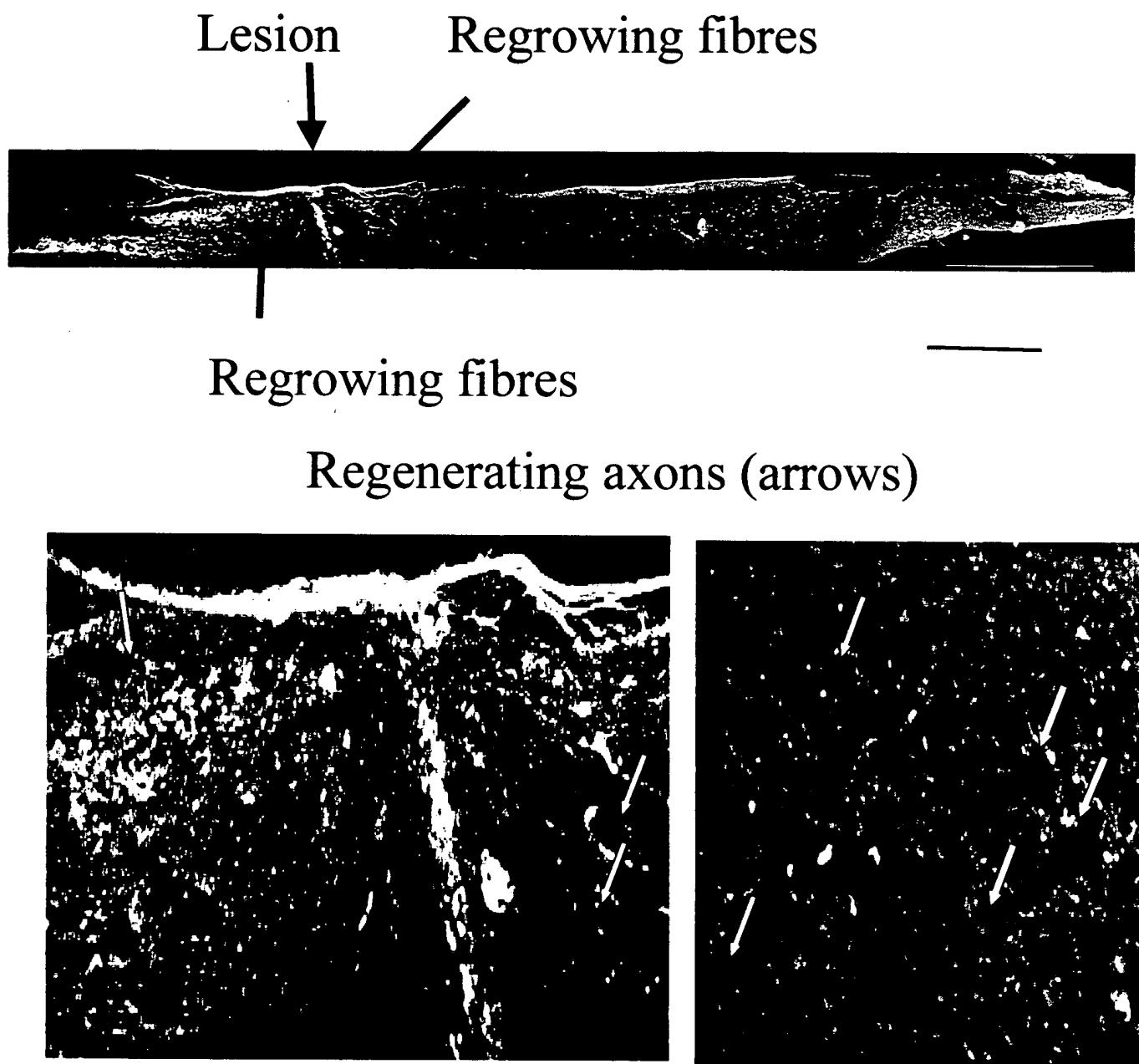


Figure 6  
C3/fibrin glue -treated spinal cord

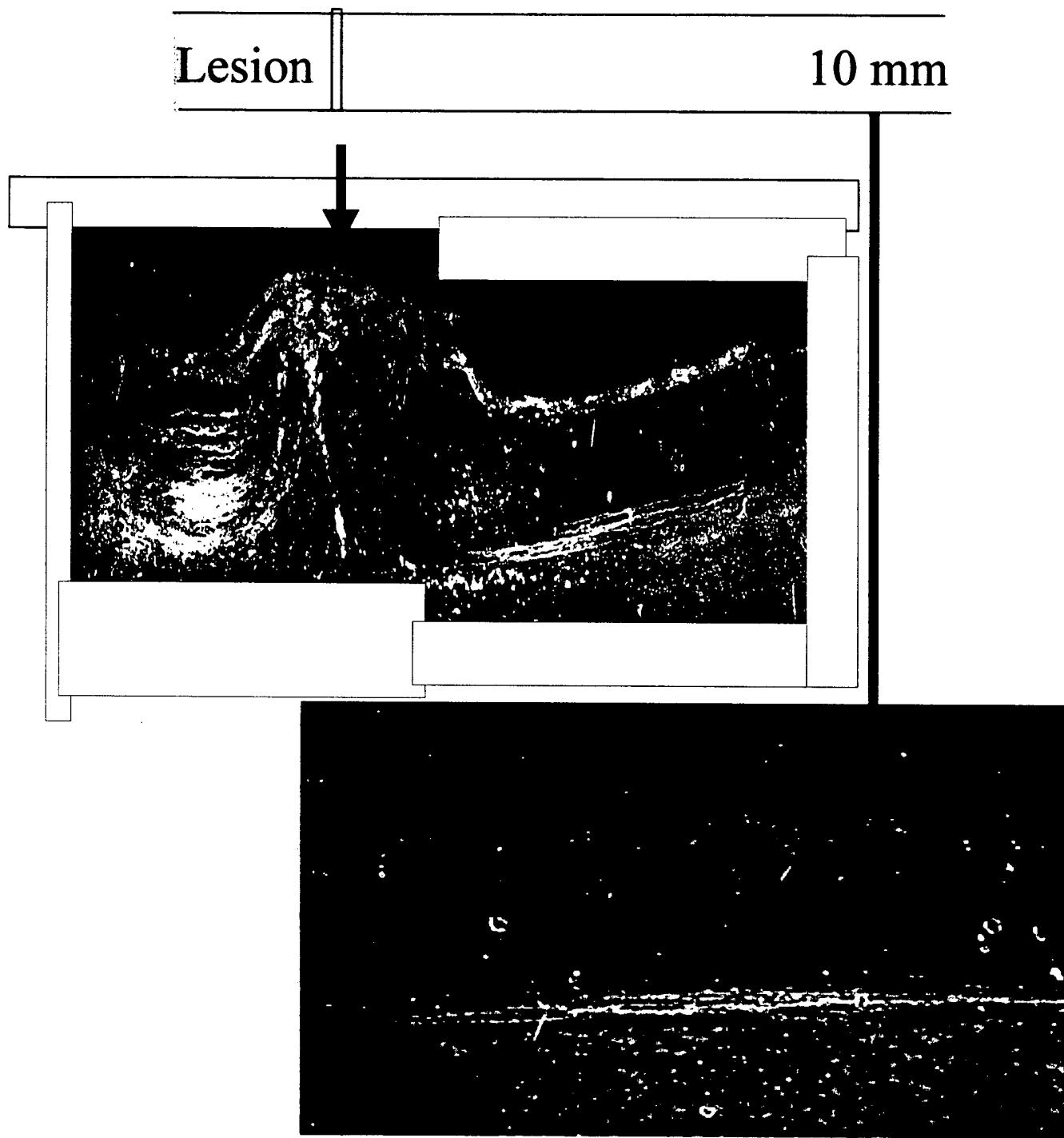
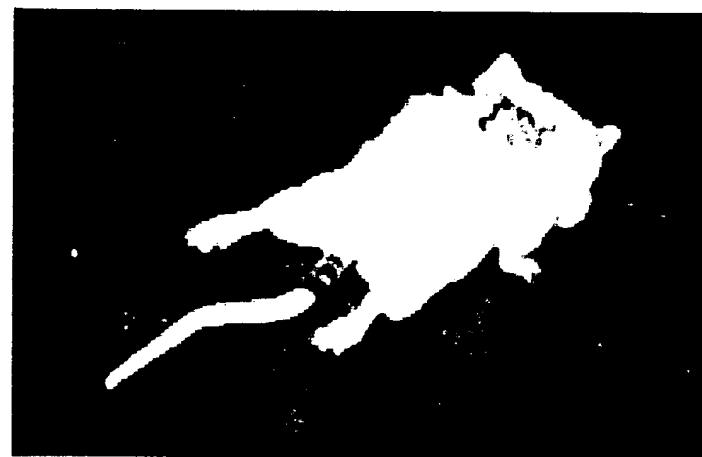


Figure 7

# Early Functional recovery

Control



C3-treated

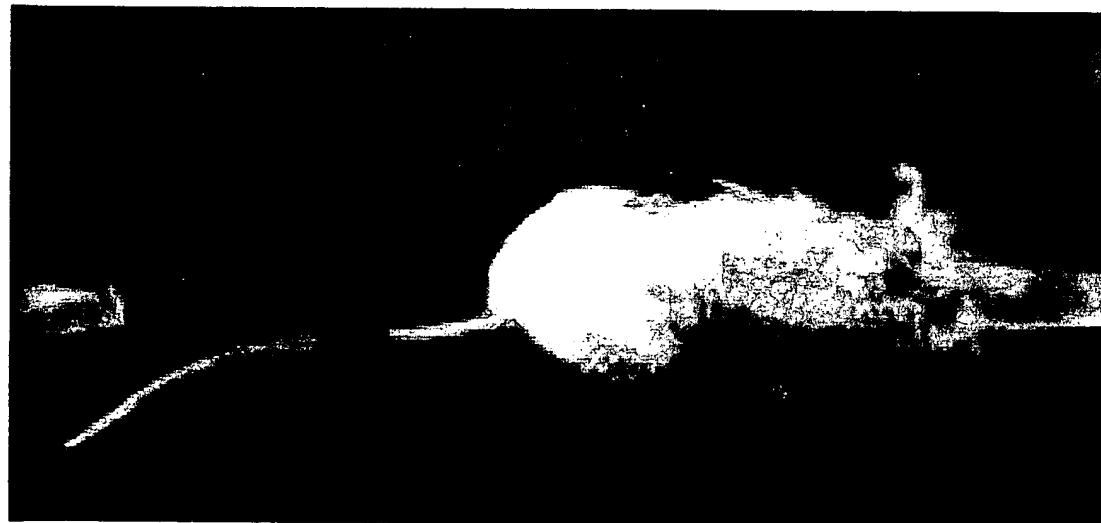


Figure 7a

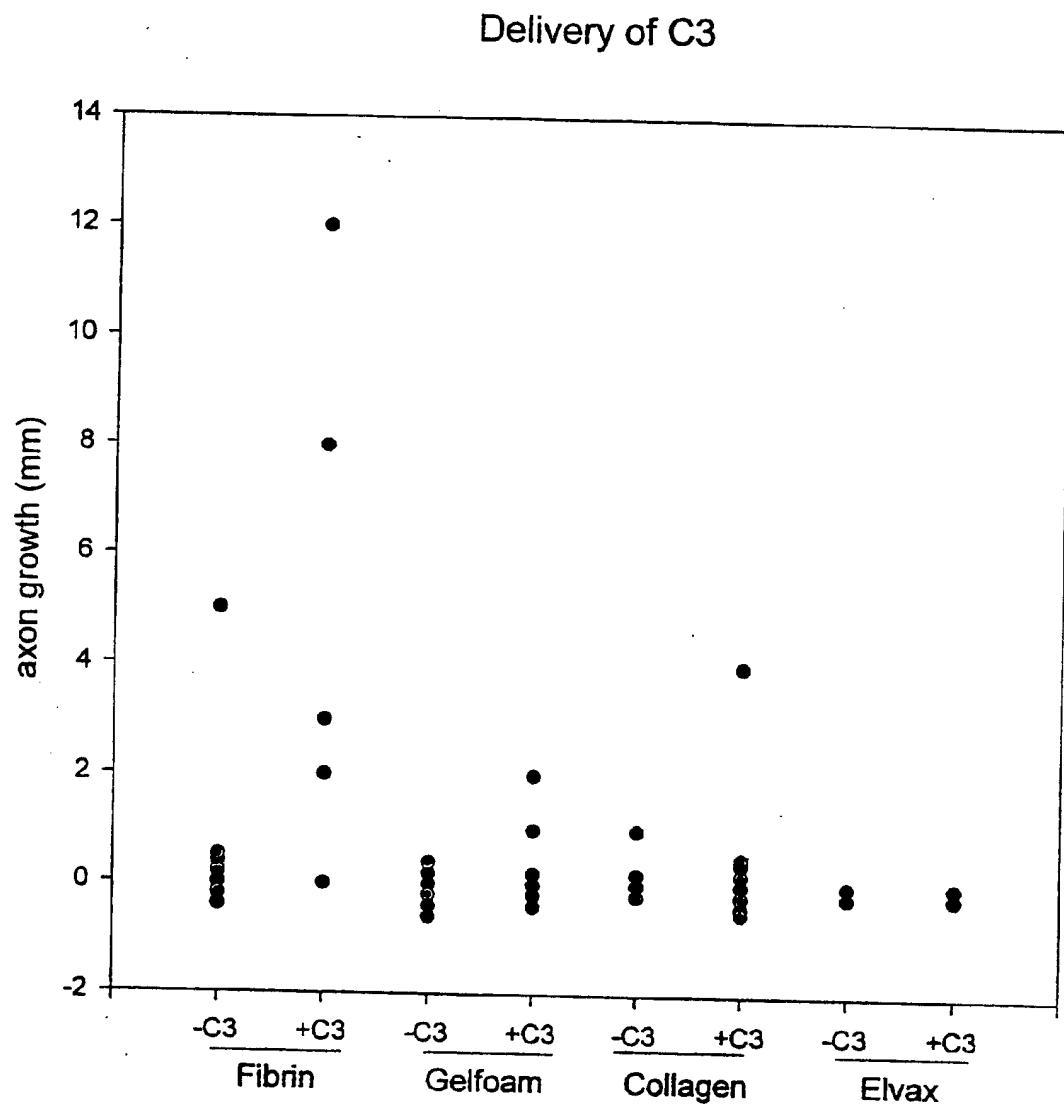


Figure 8

# BBB tests show recovery after C3 treatment

